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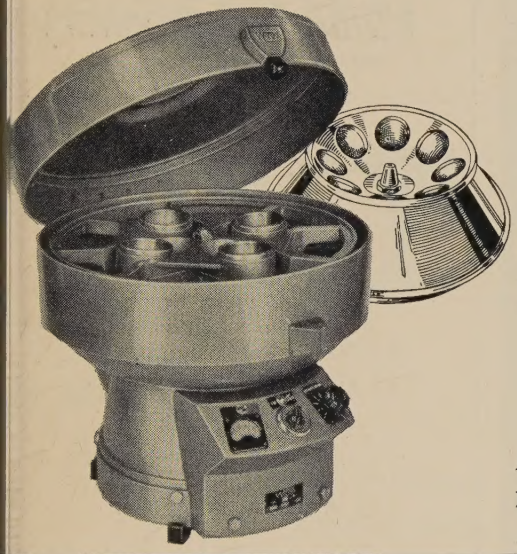

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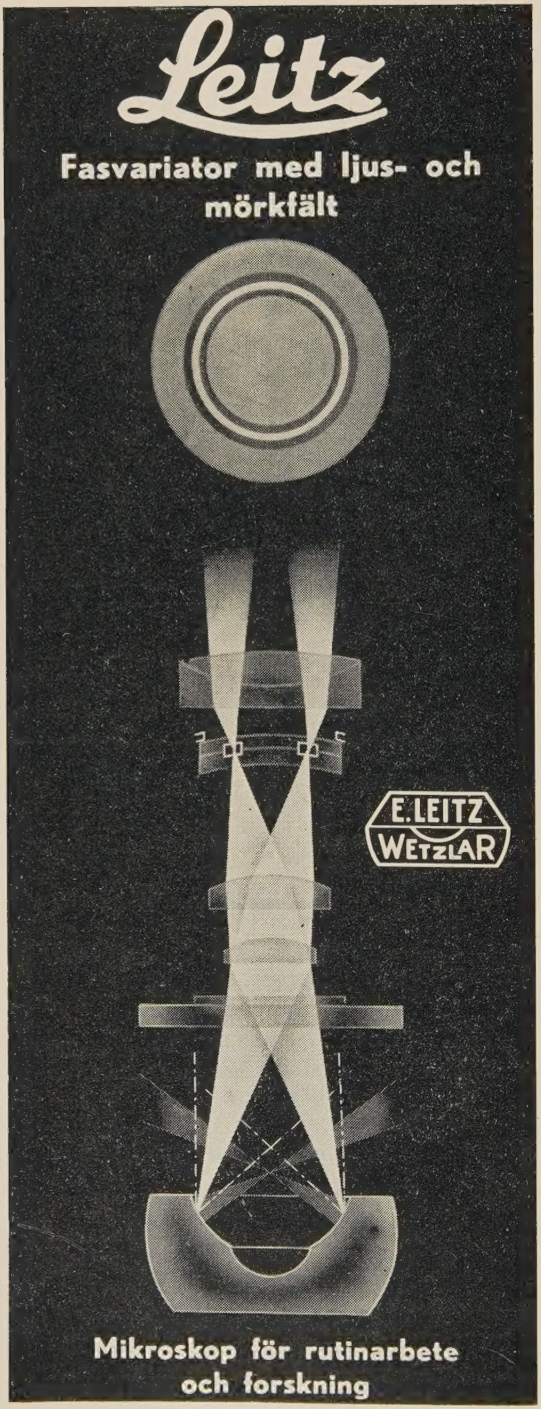

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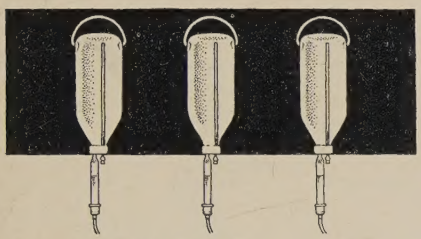

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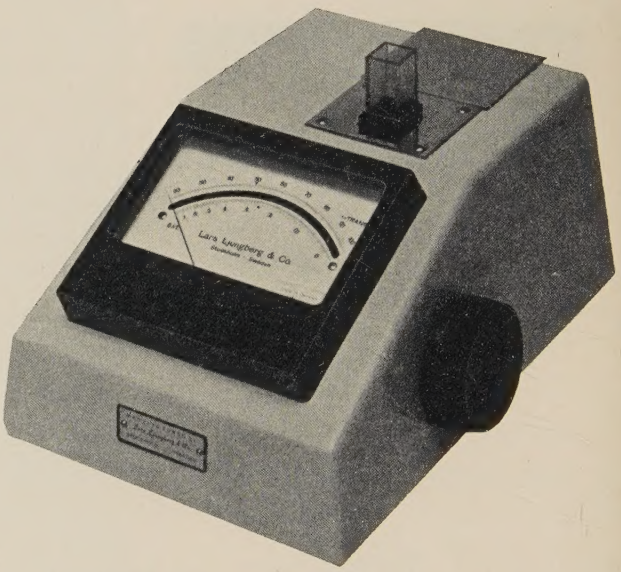

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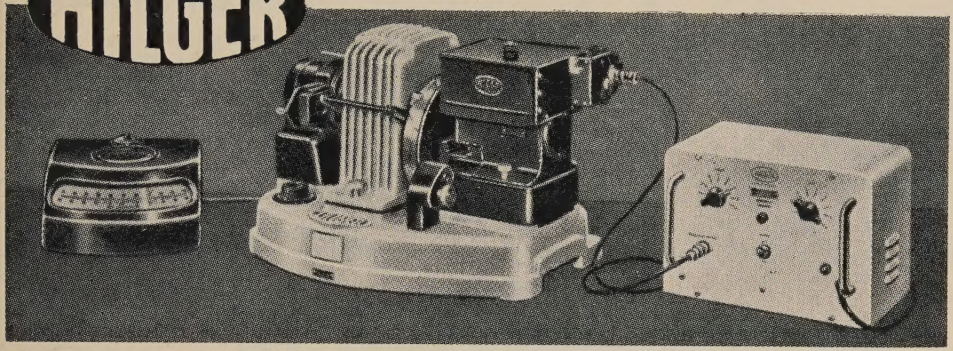

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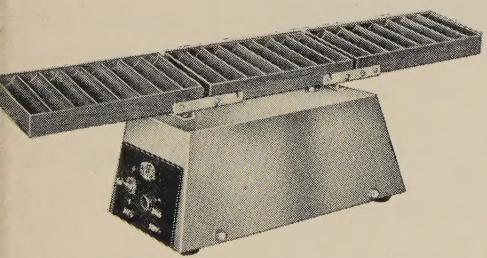

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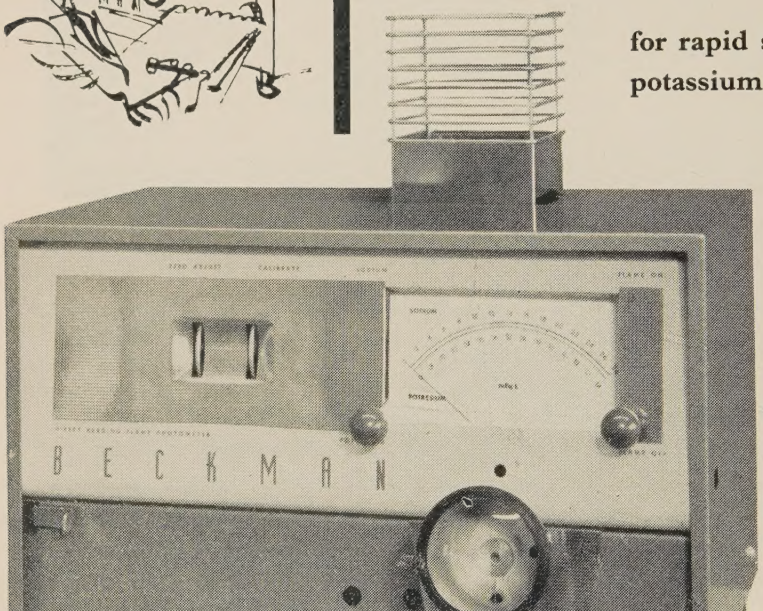

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QUANTITATIVE DETERMINATION OF BARBITAL IN BLOOD SERUM BY ULTRAVIOLET ABSORBTION SPECTROPHOTOMETRY

By R. ASKEVOLD and F. LØKEN

From The Central Laboratory, Ullevål Hospital, Oslo, Norway

(Received for publication September 9, 1955)

'he quantitative determination of barbi-ates in blood serum and other biological erials is dependent on one or more ex-tions, either with ether (Hanson, 1944; ila & Alcaide, 1948; Griffon & le ton, 1948; Giotti, 1949; Born, 1949; ld & Hine, 1949; Selwyn & Dark, 0) with chloroform (Filipov, 1946; Gold-m, 1948; Walker, Fisher & McHugh, 8; Lous, 1950) or with benzene (Raven-1946).

'he barbiturates may then be determined ctly in the organic phase (Koppanyi, e, Murphy & Krop, 1934; Cohen, 1946) after reextraction into aqueous phase alker *et al.*, 1948; Gould & Hine, 1949; n, 1949; Lous, 1950; Dybing, 1955).

'or the separation of the individual bar-rates one might use chromatography ieg, 1952; Allgén, 1955).

arbiturates have a relatively narrow ab-tion band with a maximum at 240—260 mμ in alkaline solutions. The position of the maximum is dependent on the pH. This absorbtion band is used by several authors for the quantitative determination of barbiturates (Walker *et al.*, 1948; Gould & Hine, 1949; Born, 1949; Lous, 1950).

All methods for quantitative determination of barbiturates in biological materials, which claim to give a quantitative recovery of the barbiturates present, use either continuous extractions or several repeated extractions. These methods are tedious and time consuming.

Lous (1950) uses only two extractions, one extraction from serum into chloroform, and one extraction from chloroform into alkaline buffer solution. By this method the recovery of barbiturates in blood serum is fairly poor (Dybing, 1955).

The scope of the present work is to preserve the simplification with only two ex-

tractions together with a more quantitative recovery of the barbiturates. This is done by forcing the barbiturates into the chloroform phase by the removal of water and proteins by means of addition of anhydrous sodium sulphate. In this way the volume of chloroform may be reduced (15 ml of chloroform per 3 ml serum instead of 25 ml of chloroform per 3 ml serum). As a consequence of the smaller chloroform volume, the volume of chloroform extract needed for the reextraction into the aqueous phase may also be reduced, thereby ensuring a better reextraction.

EXPERIMENTAL

Apparatus

For all extractions 25 ml glass stoppered test tubes were used. No form of lubrication was applied. The photometric readings were performed on a Hilger Uvispec spectrophotometer. The readings were carried out in matched 10 mm quartz cells.

Reagents

Sodium diemal (Pharmacopoea Norvegica).
Chloroform, redistilled.
HCl, d 1.19, ANALAR.
Na_2SO_4, anhydrous, ANALAR.
Borate buffer, pH 10.0 (Sørensen).

PRELIMINARY EXPERIMENTS

Determination of the molar extinction coefficient for sodium diemal at pH 10.0 at 239 mμ

As no reference was found in the literature on the extinction coefficient of sodium diemal at pH 10.0 at 239 mμ, a determination was made.

Sodium diemal was dissolved in borate buffer at pH 10.0 in concentrations varying from 1.10^{-4} M to 5.10^{-4} M.

Table I. *Determination of the molar extinction coefficient for diemal sodium. D_1 Light absorbtion at pH 10.0 and 239 mμ D_2: Light absorbtion in acid solution and 239 mμ.*

Conc. diemal Na	$(D_1—D_2)_{239}$ mμ	Log
$1.00 \cdot 10^{-4}$ M	0.197	3.29
$1.94 \cdot 10^{-4}$ M	0.392	3.31
$2.91 \cdot 10^{-4}$ M	0.548	3.26
$3.88 \cdot 10^{-4}$ M	0.760	3.29
$4.86 \cdot 10^{-4}$ M	0.926	3.28
$5.00 \cdot 10^{-4}$ M	1.012	3.30
Mean:		3.29

The difference in absorbance at pH 10.0 and at pH < 2 at 239 mμ was determined.

The results are given in Table I. It will be seen that the absorbance is in agreement with Beer's law for the concentrations used. Log ε was determined and found to be 3.29. This extinction coefficient is used in the following calculations for determination of the concentration of sodium diemal.

Temperature dependence

Yarborough, Haskin & Lambdin (1954) reported a decrease in absorbance with increasing temperature for several organic substances in ultraviolet light.

The temperature dependence for the absorbance of sodium diemal at pH 10.0 and 239 mμ was investigated. No difference in absorbance was observed at temperatures from 19° C to 36° C.

METHOD

15 ml of chloroform is pipetted into a test tube, 2 g of sodium sulphate is added (measuring spoon), followed by 0.1 ml HCl conc. 3 ml serum is added. The test tube is stoppered and vigorously shaken for some seconds. An additional portion of 2 g sodium sulphate is added. The test tube is stoppered and vigorously shaken for some seconds.

Sodium sulphate together with water and pro- s should by now be present as fairly hard ps. The chloroform should be free from drop- of water. If this is not the case, an additional tion of 2 g sodium sulphate should be added the test tube shaken for some seconds.

At the same time a blank is made up in the e way as the sample, with the exception that serum is added to the chloroform.

Both sample and blank are now filtered. The nels should be covered with watch glasses to vent evaporation of the chloroform. 5 ml of clear filtrate is pipetted into glass stoppered tubes. 5 ml borate buffer is added. The tubes stoppered and shaken vigorously for 3 mi- es. The tubes are centrifugated. About 3 ml of aqueous phase of the sample and of the blank transferred to 10 mm quartz cells and the ple is read in the photometer against the blank 239 mμ. 0.05 ml HCl conc. is added to the ple and to the blank. The content in the cells nixed thoroughly by means of a stirring rod plastic. The sample is again read against the nk at 239 mμ.

Calculations

Photometer reading at 239 mμ, alkaline: D_1
Photometer reading at 239 mμ, acid: D_2
$(D_1 - D_2) \cdot 10.6$ = mg sodium diemal/100 ml serum.

RESULTS

Seven sera from persons who during the 8 days had taken no form of medicine re used in these experiments. The results analyses of these sera are given in ble II. It will be seen that most of the en sera will give a small extinction at mμ when they are analysed according the described method. The analysed con- t of sodium diemal in the seven sera ch contained no barbiturates vary from —0.6 mg/100 ml expressed as sodium mal. This source of error must of course considered in the interpretation of the lysis.

Table II. *Analysed values for content of sodium diemal in sera without addition of barbiturate. D_1: light absorbtion at pH 10 and 239 mμ. D_2: light absorbtion in acid solution and 239 mμ.*

Serum No.	$(D_1 - D_2)$	Sodium diemal found, mg/100 ml	Sodium diemal content mg/100 ml
1	0.007	0.08	0.00
	0.003	0.03	0.00
2	0.026	0.28	0.00
	0.057	0.60	0.00
3	0.022	0.23	0.00
	0.026	0.28	0.00
	0.018	0.19	0.00
4	0.018	0.19	0.00
	0.020	0.21	0.00
5	0.049	0.52	0.00
	0.053	0.56	0.00
	0.040	0.42	0.00
	0.032	0.34	0.00
6	0.046	0.49	0.00
	0.043	0.46	0.00
	0.054	0.57	0.00
7	0.050	0.53	0.00
	0.049	0.52	0.00
	0.056	0.59	0.00
	0.046	0.49	0.00

Soidum diemal in varying amounts was added to the same sera. The sera were analysed according to this method. The results are given in Table III. It will be seen that the recovery of barbiturate varyies from 80 per cent to 110 per cent with an average of 96 per cent.

DETAILS OF METHOD

1. *Addition of sodium sulphate*

About 3.5 g of anhydrous sodium sulphate will combine with 3 g of water to form the heptahydrate. According to this 2 g sodium sulphate was not enough in the extraction of the serum to remove all the water. An addition of 4 g sodium sulphate proved to suffice and an addition of more sodium sulphate (*i. e.* 8 g) did not seem to

Table III. *Analysis of sera with known amounts of sodium diemal added. D_1: light absorbtion at pH 10 and 239 mμ. D_2: light absorbtion in acid solution and at 239 mμ.*

Sample No.	$(D_1—D_2)$	Sodium diemal found, mg/100 ml	Sodium diemal content, mg/100 ml
1	0.292	3.1	4.0
	0.298	3.2	4.0
	0.307	3.3	4.0
	0.320	3.5	4.0
2	0.745	7.9	8.0
	0.721	7.7	8.0
	0.695	7.4	8.0
	0.715	7.6	8.0
3	0.573	6.1	6.4
	0.601	6.4	6.4
	0.618	6.5	6.4
	0.552	5.9	6.4
4	1.072	11.4	12.1
	0.987	10.5	12.1
	0.972	10.3	12.1
	1.060	11.3	12.1
5	1.986	21.0	20.6
	2.014	21.5	20.6
	1.974	20.9	20.6
	1.984	21.0	20.6
6	0.290	3.1	2.9
	0.313	3.3	2.9
7	0.460	4.9	4.4
	0.469	5.0	4.4
8	0.558	5.9	5.8
	0.596	6.3	5.8
9	0.691	7.3	7.1
	0.696	7.4	7.1
10	0.740	7.8	8.5
	0.723	7.7	8.5

have any influence on the results. However it seemed to be most suitable to ensure a homogeneous precipitation to add 2+2 g of sodium sulphate with shaking after each addition.

2. *Addition of HCl*

to the chloroform-serum mixture had no significant effect on the results, but it was according to Born (1949) retained to increase the solubility of the barbiturates in the chloroform.

3. *pH during reextraction*

Reextractions with 0.01 N NaOH, with 0.1 N NaOH and with borate buffer at pH 10.0 were tried. It proved to be satisfactory to use the borate buffer of pH 10.0 for the reextraction. According to Lous (1950) and Dybing (1955) this pH is the most suitable for the measuring of the absorbtion of barbiturates. The borate buffer of pH 10.0 was therefore retained for the reextraction.

4. *Time of shaking during reextraction*

Experiments to increase the recovery by increasing the time of shaking during reextraction beyond the 3 minutes stated by Lous (1950) proved to be of no effect.

SOURCES OF ERROR

Sulphonamides and related compounds will give too high results (Lous, 1950; Nielsen & Prætorius, 1952; Dybing, 1955).

Salicylates will give too low results and eventually in some cases mask the presence of barbiturates (Nielsen & Prætorius, 1952; Ungar, Damgaard & Wang, 1952; Dybing, 1955) Chromatographic identification of the barbiturates might be of interest in these cases (Grieg, 1952; Allgén, 1955).

SUMMARY

A rapid and simple extraction method for the determination of barbital in blood serum is described.

The barbiturate is extracted from the serum into chloroform through a simultaneous removal of water and proteins by addition of anhydrous sodium sulphate.

The barbiturate is reextracted from the oroform by a borate buffer of pH 10.0. The amount of barbiturate is determined ctro-photometrically at 239 mμ.

The molar extinction coefficient for ium diemal at pH 10.0 and 239 mμ is ermined and used for the calculation of spectrophotometric readings.

The recovery of sodium diemal added to um varyies from 80—110 per cent with average of 96 per cent. The recovery ues of more than 100 per cent are prob- y caused by substances other than barbi- ates extracted from some of the sera.

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MATHEMATICAL AND PHYSICAL CONSIDERATIONS CONCERNING THE RESIDUAL VOLUME OF THE HEART VENTRICLES

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Investigations on the mechanism of the heart's response to loading have held the interest of cardiac physiologists for centuries. As early as the 17th century, Stephen Hales discovered the discrepancy between the average systolic discharge and the volume capacity of the heart ventricles. Roy & Adami (1888), however, were the first to prove that the residual volume of the ventricles plays a most important role in the cardiac compensatory mechanism.

Starling (1918) determined the ratio heart volume/stroke volume using heart-lung preparations. Calculations showed that this index was fairly constant in healthy hearts of varying size, but revealed increases in cases of heart failure with cardiac decompensation. Similar findings have been published by Lysholm, Nylin & Quarnå (1934) and Liljestrand, Lysholm, Nylin and Zachrisson (1939), who determined the heart volume roentgenologically using the Kahlstorff formula, and the stroke volume by Grollman's acetylene gas method. In decompensated cases Nylin and co-workers found an increase in the ratio heart volume/stroke volume from the normal 10.3 to an average of 13.5. In severe cases of cardiac decompensation the index rose to more than four times the normal. The stroke volume in these cases was only about half the normal. The fact that the total heart volume was 2.5 times the normal, together with the hardly visible pulsations found on fluoroscopic examination, led Nylin to conclude that the enlargements are not only the result of regurgitation, but also result from large residual volumes. Nylin furthermore maintained that in normal individuals the residual volume approximates the stroke volume.

Several methods for determining the circulation time have been published. Generally this is not taken to be the time required for a particle to traverse the whole cardiovascular system, but the time needed to pass from one point of the circulation to wherever registration of the indicator is most convenient. Various substances such as decholin, histamine, fluorescine and radioactive materials have been used. When the indicator is injected into the venous side of the system, the time of arrival at the place of registration determines the circulation time. In cases where the indicator passes the heart and lungs, the circulation time has been used for a very rough estimation of the residual volume of the heart (Nylin, 1943).

Using the Rohrer-Kahlstorff formula, Friedman (1950, 1951) measured the heart volume roentgenologically *ante mortem,* and

t mortem after *rigor mortis* had set in. e amount of blood remaining in the heart s then determined by the displacement hod. By substracting the *post mortem* n the *ante mortem* roentgenologically de- nined volume, and adding the heart ume estimated by the displacement hod, a figure was obtained which agreed ly well with the heart volume as deter- ed by the X-ray method *ante mortem.* this way the residual volume of blood in heart could be estimated.

Lagerlöf, Werkö, Bucht & Holmgren 49) have used the dye injection method determining the blood volume in the it side of the heart. Evans blue dye was t injected through a cardiac catheter into pulmonary artery and then at the en- ce of the superior *vena cava* in the right icle. Blood was sampled from the femoral ry. By subtracting the volumes calcu- d from the two dye curves, the volume blood in the right auricle and ventricle determined.

Because of the vagueness of the term sidual volume" it seems desirable to de- it more exactly. One cannot speak of dual volume unless one relates it to a l-defined cavity (or cavities). Whereas ventricles are well-defined, the auricles definitely not. Discussions concerning auricular residual volume are very un- r as a) the contraction of the auricular during its systole is more or less in- plete, and at least in auricular fibrilla- of minor importance for filling the ven- es, b) the blood in the auricles is in con- ous movement and c) the contraction he auricular muscle is more a peristaltic e than a concentric contraction (Wig- gers, 1949). In addition to this, the auricles and ventricles contract at different times, so that the residual volume of the whole heart should theoretically be composed of blood remaining in the auricles at the end of auri- cular contraction, and the blood in the ven- tricles at the end of the time interval between auricular systole and the beginning of the isometric realaxation of the ventricular muscle. In cardiac insufficiency, where the auricles as well as the ventricles are more or less dilated and often contain several litres of blood, the total heart volume deter- mined by the roentgenological methods is a good indicator of the state of the heart, while talk of a residual volume for the *whole heart* is confusing. The residual volume is thus only definable as the volume of blood remaining in one or both ventricles during the isometric relaxation period.

Bing, Heimbecker & Falholt (1951) have described a method for estimating the resi- dual volume of the right ventricle. A double lumen catheter is introduced into the heart, so that when the opening of the longer lumen is situated in the pulmonary artery, the opening of the shorter lumen is in the right ventricle. When the catheter is in this position, Evans blue dye is injected through the shorter lumen into the right ventricle. During each systole, blood mixed with dye will pass from the right ventricle into the pulmonary artery, from where a part of it is removed through the catheter into an appa- ratus which continuously registers the dye concentrations in the blood (Friedlich, Heim- becker & Bing, 1950). In this way a curve is obtained with a steep upstroke due to the injection of the dye and a slower downstroke due to the exponential dilution of the dye

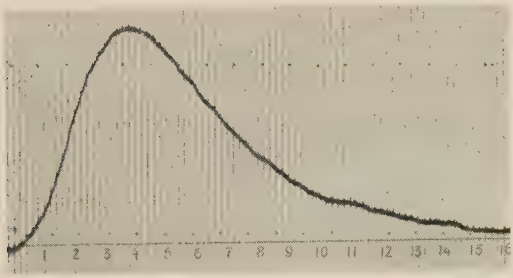


Fig. 1. Dye dilution curve obtained in an artificial pulsatile system.

in the blood (Figure 1). Plotting this curve on semilogarithmic paper (Figure 2) gives a linear dilution curve until recirculation sets in, and the cardiac output can be calculated by the Hamilton formula (Kinsman, Moore & Hamilton, 1929).

The slope of the dilution curve is determined by the stroke volume (SV), the residual volume of the right ventricle (RV), the pulse rate and the so-called catheter factor. During the passage of the blood through the catheter to the cuvette of the dye-recorder, changes in concentration in a cross section of the catheter will occur due to mixing, so that the downslope of the dilution curve tends to decrease with increased length of the conducting tubes. The catheter factor is thus defined as the factor correcting the slope of the dilution curve obtained to the theoretical slope. By examining the influence of the flow, the residual volume and the catheter factor on the slope of the dilution curves obtained in an artificial circulatory system, Bing, Heimbecker & Falholt found a linear relationship on semilogarithmic paper between the ratio flow/RV as the ordinate and the slope of the dilution curve as abscissa. The application of these findings to humans

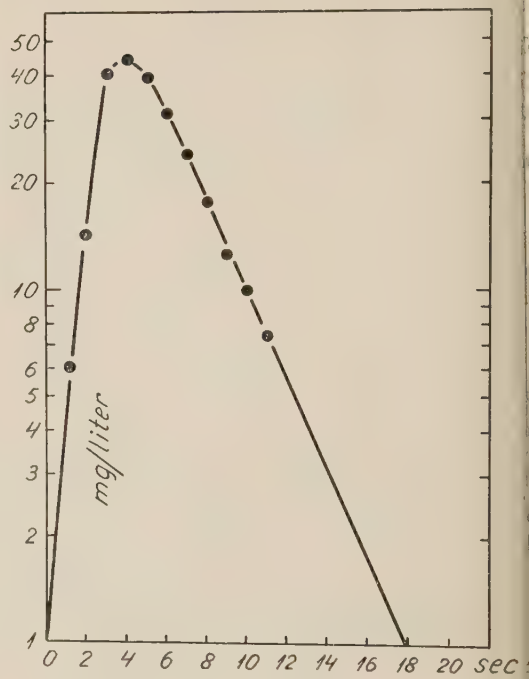


Fig. 2. The dye dilution curve plotted on semilogarithmic paper. Minute volume is calculated from the Hamilton formula: flow = mg · 60/area, where mg is the amount of dye injected, and area = area under the curve.

made possible the estimation of the residual volume of the right ventricle. However, as the slope of the dilution curve is dependent on the stroke volume and pulse rate, and as the combination of various stroke volumes with the corresponding pulse rates may give the same minute volume, it will be seen that estimates of the residual volume based upon the minute volume as calculated from the dye curve are subject to certain errors.

The present paper attempts to introduce a correction for this into the formula for calculating the residual volume of the right ventricle.

CALCULATIONS

onsidering a periodic working pump, in which greatest volume (*i. e.* when the plunger is in outer position) is called V, the fluid expelled stroke SV, and the fluid remaining in the nber RV, we have:

$$V = SV + RV \qquad (1)$$

f a certain amount of dye, I, is now injected the chamber of the pump, the instantaneous entration will be:

$$C_o = \frac{I}{V} \qquad (2)$$

or each stroke the concentration of dye in the p will decrease by the factor RV/V or, accord- to equation (1) $\frac{RV}{SV + RV} = \frac{1}{1 + SV/RV}$. The entration of dye in the pump after n periods ius given by:

$$C_n = \frac{C_o}{(1 + SV/RV)^n} \qquad (3)$$

$$\log C_n = \log C_o - n \log (1 + SV/RV) \qquad (4)$$

Equation 4 expresses the linear decrease of the rithm of concentrations of dye during n periods. ccording to equation 4 the slope of this curve is:

$$a = -\log (1 + SV/RV) \qquad (5)$$

combining aquations 3 and 5, the following ression of a is obtained:

$$a = -\frac{d \log C_n}{dn} \qquad (6)$$

ch is the slope of the dilution curve per period. Equation 5 can now be expressed as:

$$\log (1 + SV/RV = \frac{d \log C_n}{dn}$$

n natural logarithms

$$SV/RV = e^{(-d \text{ l.n. } C_n/dn)} - 1 \qquad (7)$$

quation 7 expresses the relationship between ratio of SV/RV and the slope of the dilution e per period. The curve determined by this tion is linear on semilogarithmic paper, when C_n/dn is above 1.5. When below 1.5, the curve symptotic to the ordinate axis.

The application of this formula to the human heart would make possible the calculation of the residual volume of the right ventricle using the method described earlier, if no mixing of blood occurred during the passage through the catheter. As this is the case, however, an artificial pulsatile circulatory system was constructed, in which the influence of the catheter on the dilution curve could be studied.

PHYSICAL EXPERIMENTS.

In constructing an artificial pulsatile circulatory system certain basic demands must be met:

1) The residual volume of the chamber of the pump must be variable from 50 to 350 ml. This was managed by making the bottom of the chamber displaceable, and by giving the cylindric chamber, *c*, a diameter of 4.5 cm and a length of 30 cm.

2) The stroke volume must be variable from 10 to 100 ml. This was obtained by making the fixation of the piston rod variable in relation to the centre of the rotating disc, *d*, and the measures of the chamber are as mentioned under 1).

3) The dye is injected through a stop-cock into the chamber immediately beside the inflow valve, *i*. In order to assure that the dye mixed sufficiently well with the fluid in the chamber, the outlet valve, *o*, was built into the bottom of the chamber, and the inflow opening was made displacable in relation to the bottom position of the plunger. The importance of this will be seen later.

4) The inflow and outflow valves must be constructed so that regurgitation is excluded.

5) The outflow tube, *t*, must be constructed so that the tip of the catheter conducting the mixture of dye and fluid to the dye-recorder can be placed immediately above the outflow valve, and so that the fluid passing through the chamber can be collected and measured.

6) It must be possible to observe the mixing of the dye with the fluid in the chamber directly. This was achieved by constructing the cylinder of the pump out of plexiglass.

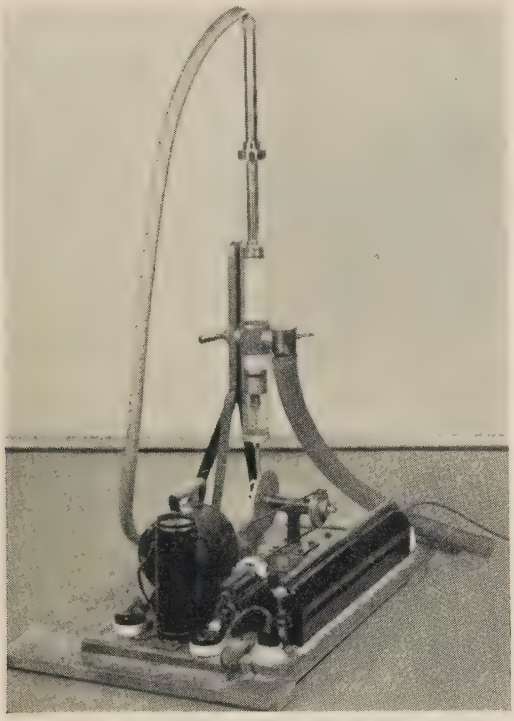


Fig. 3. The artificial pulsatile system.

7) The pump must be able to work at from 60 to 100 cycles per minute.

The pump illustrated in Figures 3—4 fulfilled all these criteria.

In the present series of experiments water was used as the circulatory fluid. The dye concentrations were registered by means of a dye-recorder constructed by Falholt & Kaiser (1955). The minute volume was directly measured and compared to that calculated by the Hamilton formula. The water was conducted to the dye-recorder through the longer lumen of a double lumen catheter from the U. S. Catheter and Instrument Company.

A dye curve from the artificial system is illustrated in Figure 1. The directly measured minute volume and that calculated from the dye curves are compared in Figure 5.

The figures obtained from the artificial circulatory system were plotted on semilogarithmic paper, the ratio SV/RV being the ordinate and

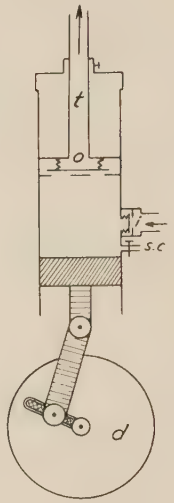


Fig. 4. Sketch of the artificial system. i = inflow valve. o = outflow valve. d = rotating disc. s.c. = stopcock for injection of dye. t = outflow tube.

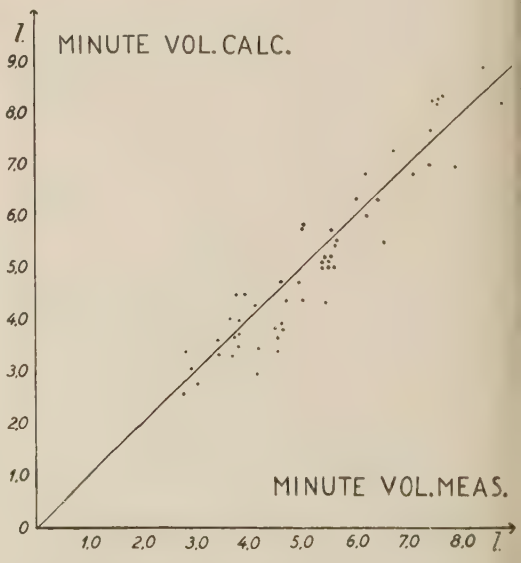
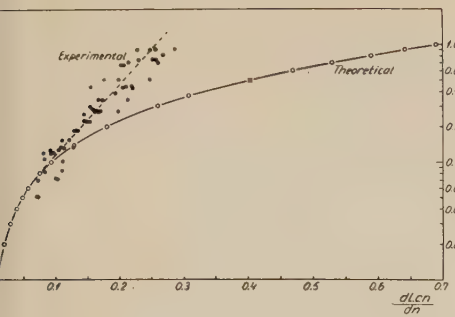


Fig. 5. Comparison of the minute volumes directly measured, and those calculated from the dye curves. The variation of the points from the theoretical curve was below 13 per cent.



6. Data from the artificial pulsatile system, ing the relationship between the ratio SV/RV d l.c/dn, the fall in logarithmic concentrations e per stroke, plotted on semilogarithmic paper. variation of the points from the average line s below 20 per cent. a = theoretical curve. b = experimental curve.

/dn the abscissa (Figure 6). d l.c/dn was cal- ed from the equation:

$$\text{d l.c/dn} = \frac{(\text{l.C}_1 - \text{l.C}_2) \cdot 60}{(t_2 - t_1) \cdot \text{freq.}}$$

e C_1 and C_2 are two arbitrarily chosen con- ations on the dye curve, t_1 and t_2 the corre- ding time values, and freq. the number of es per minute.

will be seen that the curve for SV/RV and /dn approximated a straight line. It is to be cted that this curve is also asymptotic to the ate axis at its lower limits. As the mixing e dye with the water flowing in the system satisfactory in this area, it was not possible etermine the curve here. However, the ratio V/RV below 0.07 is probably below physio- al limits.

DISCUSSION

he application of the experimentally loped formula for calculating the residual me of humans is dependent on mixing dye satisfactorily with the water in the ing chamber of the pump. It was easy bserve that the position of the inflow opening in relation to the inner position of theplunger and to the outflow valve was of vital importance for effective mixing. Before single dye curves were obtained the inflow opening was therefore adjusted to the position which apparently gave the best results, being about one third the way up the wall of the chamber, when the plunger vas at its innermost position. When the inflow opening had been so adjusted, the mixing was apparently perfect when relatively small residual volumes were present. In order to obtain good mixing even with large residual volumes (250—300 ml), a propeller was fixed to the inner surface of the plunger.

In order to observe the result of bad mixing, the inflow opening was placed im-

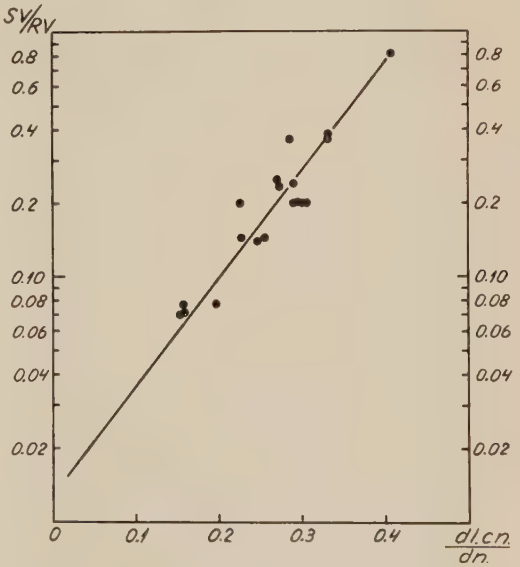


Fig. 7. Data from the artificial system, when the mixing of dye with the water flowing through the chamber was intentionally bad. It may be seen that in this case a linear relationship between SV/RV and d l.c/dn on semilogarithmic paper was obtained.

mediately above the site of the inner position of the plunger. It was easily observed that the mixing was poor, and that the dye was expelled from the chamber after a few strokes of the pump. In spite of this, the dye curves obtained showed an exponential dilution, but the slope of the curve was steeper than was to be expected with the present systolic and residual volumes, and the flow calculated agreed only poorly with that directly measured. As seen in Figure 7 the relationship between SV/RV and d l.c/dn was linear on semilogarithmic paper even in this case. In other words, the fact that a linear relationship is found is no indication of good mixture in the chamber.

According to the above, the exponential curves obtained in humans for residual volume determinations using the method described earlier are not indicative of good mixing in the ventricle. If the mixture of dye and blood in the right ventricle is not perfect, this means that pools of blood remain stagnant in the ventricle. Consequently the residual volume determined by the present method must be considered too small. In Bing's method, the dye is sprayed through the 5 openings of the shorter lumen of the catheter into the right ventricle, and the dye-blood mixture may be perfect. Assuming that pools of blood mixed with dye are retained in the right ventricle, a part of the dye injected will remain in the ventricle. If Hamilton's formula is used, the cardiac output will then be overestimated, and the subsequent calculation of the residual volume will likewise give a spuriously high figure.

Whether one considers dye and blood to be mixed perfectly in the ventricle is a matter of persuasion. Especially where there are very large residual volumes (600—700 ml) the belief will have to be questioned, and the figures obtained by Bing's method are minimal values. In the human heart, however, where blood passes from the auricle through the tricuspid valve into the irregularly shaped ventricle, furnished with trabecles, and where the blood changes direction about 160°, the flow must undoubtedly be very turbulent, which makes perfect mixing of the dye with the blood in the ventricle probable.

The influence of the catheter factor on the relationship between the ratio SV/RV and d l.c/dn is illustrated by curves *a* and *b* in Figure 6. It will be seen that the influence of the catheter increases as the ratio SV/RV rises. Thus the catheter factor must necessarily be corrected for, for each type of catheter used, using fluid of the same viscosity as whole blood at 37° C. Furthermore, when Bing's method is applied to humans, the suction of blood through the catheter must not exceed the rate where laminar blood flow is changed into turbulent flow.

The relatively large deviations of certain points from the average curve *b* in Figure 6 is possibly attributable to insufficient mixing of the dye with the water in the chamber of the artificial system. If this is the case, and the mixing of dye with blood in humans is prefect, the error of residual blood volume determinations in the right ventricle is below 20 per cent.

SUMMARY

Based upon the method for estimating the residual volume of blood in the right heart ventricle published by Bing, Heimbecker & Falholt, a new formula for calculating the residual volume is proposed.

formula expressing the relationship be-
en the ratio SV/RV and the fall in loga-
mic concentrations of Evans blue dye in
pulmonary artery after injection of the
into the right ventricle has been devel-
d.

By means of a mechanical pulsatile circu-
ry system, where the stroke volume, resi-
l volume and the stroke frequency can
varied independently, a correction for the
mixing occurring during the sampling of blood through a cardiac catheter has been established.

The approximation of the relationship between SV/RV and the fall in logarithmic concentrations of dye per heart beat to a straight line is thus mathematically and physically proved.

The errors of the method are discussed.

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SEPARATION OF LEUKOCYTES

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The discovery of leukocyte antibodies in the serum of patients with leukopenia has actualized the need of a simple method for separation of leukocytes from human blood. Such a method may be valuable also for morphological and biochemical investigations of leukocytes. The ideal suspension of leukocytes has to be free from foreign substances, red cells and platelets, and the leukocytes must be undamaged and unagglutinated. As may be sen from Table I, most methods recently used do not fulfil these requirements. The suspension obtained by Tullis (1953) is probably relatively fre from red cells and platelets, but he does not say whether it is completely so. The special centrifuge required limits the use of the method to certain special laboratories. According to Vallee, Hughes & Gibson (1947), and to Singer, Silberbach & Schwartz (1947), the suspensions obtained by their respective methods are not homogeneous enough for accurate counting. Hence, they are not satisfactory.

METHODS TESTED

As suggested by Dausset, Nenna & Brecy (1954), defibrinated blood was used, and the sedimentation rate of the red cells increased by addition of Plasmosan (3.5 per cent polyvinylpyrrolidone). All the glassware was siliconized, exept the glass beads for defibrination, and the procedure carried out under aseptic conditions. About 50—60 ml of venous blood was drawn into an Erlenmeyer flask containing glass beads, and the flask rotated for about 15 minutes. About 10 ml of the blood was removed for separation of serum, the remainder poured into a gradated glass cylinder, and ⅛ per volume of Plasmosan added. After mixing in another bottle, the mixture was pipetted into 12 ml centrifuge tubes, taking care that the inner wall of the tubes above the surface remained clean. The tubes were then stopped an incubated at 37° C, standing at a 60° angle to the horisontal plane. After 45 minutes the upper two-thirds of the serum layer was pipetted off, using a Pasteur pipette, into one or several centrifuge tubes. These were cooled off in a refrigerator and centrifuged for 5 minutes at 1,500 rpm under cool conditions. If a cold centrifuge is not available, it is quite satisfactory to use an ordinary centrifuge with large cups half filled with ice. The supernantant was removed and the sediment resuspended in saline or Plasmosan-free serum.

If a completely red cell-free suspension of leukocytes was wanted, the procedure was slightly modified. After sedimentation at 37° C, saponin solution was added to the supernatant, the hemolysing power of the saponin used having previously been titrated. In most instances, about 1/20 per volume of a 10 per cent solution in distilled water had to be added to lyse all red cells remaining in the serum. After half an hour at room temperature, the suspension was cooled and centrifuged as mentioned before. Ten times less, *i. e.* 1/20 per volume of a 1 per cent solution of saponin, was sufficient if the saponin was added after centrifugation and

* Aided by a grant from the Sigrid Juselius Stiftelse.

able I. *The presence of red cells, platelets and foreign substances in the leukocyte suspension produced by different methods.*

Authors		Red cells	Platelets	Anticoag.	Other substances
nin & Schwartz	(1954)	+	+	Heparin	—
unsteiner, Pakesch & Vetter	(1953)	+**	+	Heparin	Polyvinylpyrrolidone
sset, Nenna & Brecy	(1954)	+	—	—	Polyvinylpyrrolidone
dsmit & van Loghem	(1953)	+	—	—	Dextran
scher	(1954)	+**	+**	Heparin*	Fibrinogen, Triton
or & Burnett	(1948)	+	+**	Heparin	Fibrinogen
schlin & Schmid	(1952)	+	+	Heparin	Polyvinylpyrrolidone
tensson & Vikbladh	(1945)	+	+	Heparin	
er, Silberbach & Schwartz	(1947)	—	+**	Heparin	Lysolecitin, Gramicidin
is	(1953)	—**	—**	—	Dextran*
ee, Hughes & Gibson	(1947)	—	+	Heparin	Albumin
astjerna	(1954)	+	+	Sequest-rene	

* Later removed by centrifugation and washing.
** Not clearly stated by the authors

spension of the leukocytes in saline. After 30 utes at room temperature, the suspension was ed and centrifuged a second time at 1,500 rpm 5 minutes, the supernatant removed, and the resuspended in saline or serum.

igas & Osgood (1954) have suggested the of phytoagglutinin to enhance the sedimenta- rate. This method was tested, using a con- ration of 0.1 mg phyto-agglutinin per ml of d. The concentration was more than ten s stronger than the titer limit when whole d was used for the titration, but in spite of ng agglutination, the sedimentation rate was satisfactory. The red cells settled more rapidly ne blood was diluted with the same amount of ne before the addition of phyto-agglutinin. How- , the sedimentation of red cells was less com- than with the use of Plasmosan.

RESULTS AND COMMENT

Defibrination of the blood removes the elets and renders it possible to avoid coagulants, but the leukocyte count is inished by the defibrination, probably be- se some leukocytes stick to the fibrin clot. en leukocyte counts were performed be- and after defibrination, their number was nd to be about 20—30 per cent less after defibrination. After addition of Plasmosan and sedimentation for 45 minutes at 37° C, the leukocyte count in the serum was about 20 per cent less than that in the defibrinated blood. About 15 ml of serum was usually obtained from 50 ml of defibrinated blood. Hence, the recovery of white cells was about ⅙ of all the white cells in the blood drawn from the donor.

The effects of Plasmosan and of Macrodex on the sedimentation of red cells were compared and found to be about the same. Both of these substances enhanced the sedimentation rate more effectively than did phyto-agglutinin in the concentration tested in the present experiments. It seems to be a matter of taste whether Plasmosan or Macrodex is used. However, if saponin is added to lyse the red cells, Macrodex cannot be used, because it was found to inhibit the saponin hemolysis to a marked degree.

The addition of saponin, in the above mentioned concentration, did not affect the leukocyte count, neither did the centrifugation under cool conditions.

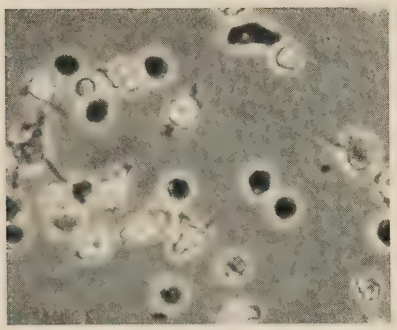

Fig. 1. Suspension of leukocytes and a few red cells. No saponin used. × 400. Phase contrast.

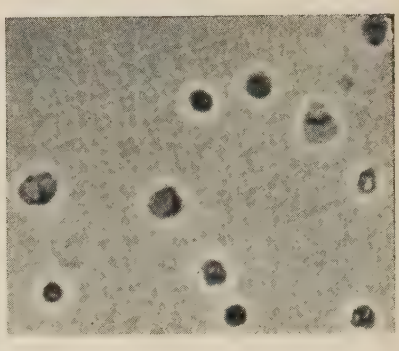

Fig. 2. Suspension of leukocytes after saponin hemolysis of red cells. × 400. Phase contrast.

The vitability of the leukocytes of the suspension obtained, was estimated by study under a microscope with ordinary and phase contrast optics. If no saponin had been added during the procedure, the leukocytes of the suspension looked quite normal and undamaged for several hours. They showed normal ameboid movement, and the granules were in lively motion (Fig. 1). They maintain their motility equally in saline as in serum and, if the remaining traces of Plasmosan are to be removed, the leukocytes may be washed with saline. When saponin was used, the leukocytes seemed to be damaged to a varying degree. Sometimes most leukocytes still looked normal, but some of them had shrunk, and small clumps of leukocytes were always seen. The damage seemed to be more severe if saponin was added to cells suspended in saline, even if the concentration was lower than when added to leukocytes in serum. The motility of the cells was poor, and the nuclei of many cells were swollen (Fig.s 2 and 3).

The use of siliconized glass ware was found essential, not for the preservation of leukocytes, but for a more complete sedimentation of the red cells, because a smaller number of erythrocytes stick to the glass in the serum layer if the glass is non-wettable.

After centrifugation and resuspension in saline or serum, the leukocyte count may be standardized. The present author found a suspension of 10,000 leukocytes per mm^3 suitable when used for the demonstration of leukocyte agglutinin in patient sera. For this purpose it is not necessary to remove all red cells, and the saponin technique is not applicable owing to some tendency of the leukocytes to stick together. However, if the tubes are siliconized and, after sedimentation,

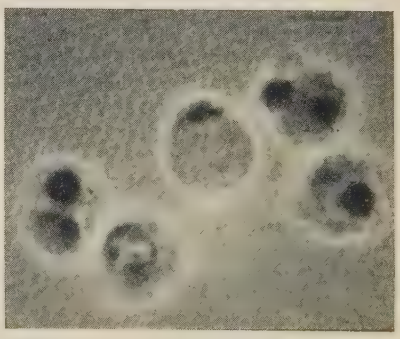

Fig. 3. Leukocytes damaged by saponin. × 1000 Phase contrast.

more than ⅔ of the serum layer is care- pipetted off, the suspension obtained n contains less red cells than leukocytes, this suspension may well be used to onstrate leukocyte agglutinins in sera. suggested by Dausset, Nenna & Brecy 54), the remaining red cells may be lysed acetic acid before reading the agglutina- test, but it is by no means necessary if number of red cells is equal to, or less the number of white cells.

SUMMARY

Methods for separation of leukocytes from od were tested as to the quantitative re- ery of cells, the purity of the suspensions, the viability of the leukocytes. By the hod of Dausset, Nenna & Brecy (1954), using siliconized glassware, the recovery white cells was about ⅛, and the final pension contained the same amount of cells as leukocytes, or slightly less, and platelets. The white cells seemed to be amaged, and their motility was normal. remaining red cells may be removed by onin hemolysis without affecting the num- of white cells, but the procedure damages leukocytes.

ACKNOWLEDGMENT

he author is indebted to Mr. Kaarlo Koljonen ne Laboratory of Biochemistry, University of inki, for the preparation of phyto-agglutinin.

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SERUM CONCENTRATIONS AFTER ORAL ADMINISTRATION OF PHENOXYMETHYLPENICILLIN ACID AND PHENOXYMETHYLPENICILLIN POTASSIUM

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The absorption of penicillin administered orally is irregular (Valentine & Shooter, 1954). This is why the oral route is not usually employed, except for infections due to very sensitive organisms and for prophylaxis.

One of the most recent penicillin preparations for oral administration is phenoxymethylpenicillin, or V penicillin, commercially available both as an acid and as potassium salt. Behrens discovered phenoxymethylpenicillin as early as 1948 but it assumed clinical importanse only after Brandl, Giovannini & Margreiter had found in 1953 that it retained its effect at very low pH *in vitro* and were able to show (1954) in both human and animal experiments that it was appropriate for oral use.

Phenoxymethylpenicillin proceeds undecomposed from the stomach to the duodenum where it is dissolved and absorbed rapidly (Spitzy, 1953; Giovannini, 1954). After the administration of 1000 units/kg of phenoxymethyl penicillin *per os*, 0.2 units/ml were found (Grandl *et al.*, 1953) in the serum within half an hour and 0.3 units/ml after one hour; subsequently the serum concentrations gradually decreased, leaving 0.16 units/ml in the blood after three hours. After four hours no appreciable amounts of penicillin were found in the serum. A comparison of the serum levels of phenoxymethylpenicillin and benzyl penicillin showed (Sjöberg, Brante & Rigner, 1955) that phenoxymethylpenicillin gives twice as high concentrations as the corresponding amount of benzyl penicillin. As the antibiotic effect of phenoxymethylpenicillin is approximately the same as that of G penicillin (Kylin, 1955) and no differences obtain in toxicity, it seems that in phenoxymethylpenicillin we have a useful preparation for oral administration.

PRESENT INVESTIGATIONS

We have studied the blood levels of phenoxymethylpenicillin in hospital patients. Penicillin concentrations obtained with phenoxymethylpenicillin acid and phenoxymethylpenicillin potassium were compared and these substances were also studied for differences between preprandial and postprandial administration.

The patient material, which was entirely unselected, comprised male and female bedridden adults of the Medical Department of Maria Hospital. Tablets containing 100 000 units of phenoxymethylpenicillin acid each or 100 000 units of phenoxymethylpenicillin potassium, respectively were used. The initial dose was only one tablet per patient. But it soon transpired that the con-

ations in the serum varied to such an extent the lowest serum concentrations could not be mined by our method. For this reason the e dose was made 300 000 units. To observe rate of absorption, serum concentration level duration, 10 patients were given 300 000 units phenoxymethylpenicillin acid on a fasting ach 2 hours before a meal. The serum samples taken after ½, 1, 2 and 4 hours. A similar edure was followed with another 10 patients were given 300 000 units of phenoxymethyl- illin potassium.

ne blood levels of penicillin were determined by lisc plate method. The culture medium con- l of Difco streptomycin assay agar (pH 7.4) the test organism of penicillin-sensitive *Sar- lutea* strain. The sterilely drawn blood samples allowed to stand at room temperature for an and centrifuged. A paper disc 15 mm in eter was dipped in the serum thus separated, cd to dry in the air and placed on an in- ted agar plate. After a 24-hour incubation ° C the diameter of the zone of inhibition was ured. By comparing its size with the zones hibition of the known penicillin concentrations e so-called standard series, the penicillin con- ation of the serum involved could be computed. rent standard series were prepared for both phenoxymethylpenicillin acid and phenoxy- ylpenicillin potassium as the *Sarcina lutea* employed proved to be slightly more sen- to the latter. The penicillin dilutions of the lard series could be made in physiological e as it was found that the addition of serum ot affect the sensitivity of the test organism

he results are given in Fig. 1.

can be seen that both phenoxymethyl- cillin acid and phenoxymethylpenicillin ssium give high serum concentrations in half an hour. The maximum level was hed in one hour. Subsequently the es gradually decline to minimal blood ls after 4 hours. Although the curves of two penicillins are practically the same,

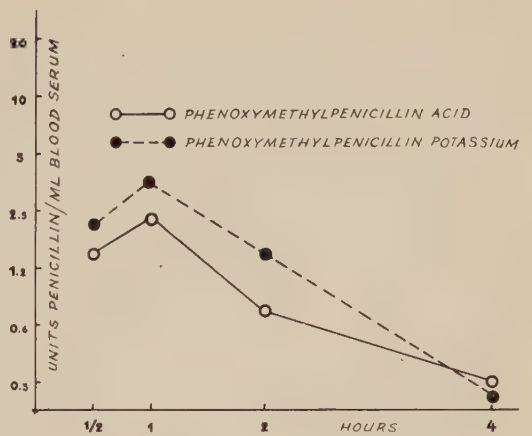

Fig. 1. Concentration of penicillin in serum at different times following administration of 300 000 units of phenoxymethylpenicillin acid (average of 10 cases) and phenoxymethylpenicillin potassium (average of 10 cases).

phenoxymethylpenicillin potassium seems to give higher values.

The effect of meals on the blood levels of phenoxymethylpenicillin was studied with 120 patients, divided into groups of 30. One of the groups was given 300 000 units of phenoxymethylpenicillin potassium an hour before the meal on a fasting stomach, another the same dose immediately after a meal; the other two groups were treated similarly with phenoxymethylpenicillin acid. The blood samples were drawn 1 hour after penicillin administration. It can be seen from Table I that phenoxymethylpenicillin potassium gave slightly higher values than phenoxymethyl- penicillin acid, and that postprandial adminis- tration of the phenoxymethylpenicillin acid in particular gave lower values. This is also indicated by the mean values for each group.

Table I shows that the individual values varied greatly with both penicillins in each of the groups.

Table I. *Penicillin concentrations, units/ml, in the serum 1 hour after the oral administration of 300 000 units.*

Phenoxymethylpenicillin potassium				Phenoxymethylpenicillin acid			
On fasting stomach		After a meal		On fasting stomach		After a meal	
Patient No.		Patient No.		Patient No.		Patient No.	
1	0.25	31	0.25	61	0.25	91	0.25
2	1.25	32	0.6	62	0.8	92	0.25
3	1.25	33	0.7	63	0.8	93	0.25
4	2.0	34	1.0	64	1.25	94	0.25
5	2.0	35	1.25	65	1.25	95	0.25
6	2.5	36	1.25	66	1.25	96	0.6
7	2.5	37	1.25	67	1.25	97	0.6
8	2.5	38	1.25	68	1.5	98	0.6
9	2.5	39	1.25	69	2.0	99	0.6
10	2.5	40	1.25	40	2.0	100	0.6
11	2.5	41	1.25	71	2.0	101	0.6
12	2.5	42	1.5	72	2.5	102	0.7
13	3.0	43	2.0	73	2.5	103	0.9
14	4.0	44	2.0	74	4.0	104	0.9
15	4.0	45	2.0	75	4.0	105	0.9
16	4.0	46	2.0	76	4.0	106	1.0
17	4.0	47	2.5	77	4.0	107	1.25
18	4.0	48	2.5	78	4.0	108	1.25
19	5.0	49	2.5	79	4.0	109	2.0
20	5.0	50	2.5	80	5.0	110	2.0
21	5.0	51	2.5	81	5.0	111	2.5
22	5.0	52	4.0	82	5.0	112	2.5
23	5.0	53	5.0	83	5.0	113	2.5
24	5.0	54	5.0	84	5.0	114	2.5
25	5.0	55	5.0	85	5.0	115	3.0
26	8.0	56	5.0	86	5.0	116	4.0
27	8.0	57	6.0	87	7.0	117	4.0
28	10.0	58	8.0	88	8.0	118	5.0
29	10.0	59	10.0	89	10.0	119	5.0
30	10.0	60	10.0	90	10.0	120	5.0
Average	4.3		3.0		3.8		1.7

SUMMARY AND CONCLUSION

Phenoxymethylpenicillin potassium and phenoxymethylpenicillin acid are absorbed rapidly, and a high concentration is obtained in the blood within half an hour. The maximum is reached in one hour. Within four hours the values obtained with the two penicillins are practically of the same order, for phenoxymethylpenicillin potassium slightly higher. The individual values varied greatly.

Phenoxymethylpenicillin administered on a fasting stomach gave higher penicillin concentrations in the serum than when administered postprandially. Phenoxymethylpenicillin acid in particular gave lower blood levels when taken after food. Thus the preprandial administration of phenoxymethylpenicillin seems to be indicated as recommended earlier for other penicillins (Binns 1953).

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ANTITATIVE DETERMINATION OF THE GLUCOPROTEIN TTERN OF NORMAL SERUM AFTER ELECTROPHORETIC SEPARATION ON FILTER PAPER

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(Received for publication September 22, 1955)

öw & Grönwall (1952) described a od for locating and assessing the serum proteins after electrophoretic separation filter paper medium, oxidation with dic acid, and subsequent staining with in sulfite. Björnesjö (1955) recently shed a detailed description of the tech- and an analysis of the error of the od. All of the glucoproteins studied ed with an intensity varying with the amount of protein-bound polysaccha- and variation in the composition of olysaccharides was found to be of minor rtance. These observations were made e analyses of glucoproteins with hexose, samine and sialic acid in varying pro- on.

ese investigators estimated the distribu- of the various components photometri- The accuracy of photometric determi- ns is impaired by the fairly strong color e background *i. e.*, the paper strips. If arious fractions studied are to be com- with regard to their mobility, direct ometry is preferable to elution of the On the other hand, in quantitative mination of the distribution of different onents the elution technique is prefer- because of the unavailability of photo- instruments with satisfactory accuracy.

We have devised a method for quantitative elution of the dye from the various paper segments, this technique being considered the most suitable for routine analysis of the glucoprotein pattern.

METHODS

Electrophoretic separation.—The electrophoretic technique was described in a previous paper (Laurell, Laurell & Skoog, 1955). Serum sample: 0.02 ml. Separation distance: 9 cm. Drying: at 110° C for 10 minutes.

Staining. — Staining technique according to Björnesjö (1955), but modified in the following respects:

Sulphite rinse solution: 8 g of potassium metabisulphite was used instead of 4 g.

Step 3. Rinsed twice in 70 per cent ethanol instead of once.

Between step 5 and step 6 the paper is dried between sheets of filter paper.

Step 8 is omitted.

A number of elution procedures using varying solvents, acids and bases were tried, but only the technique described below gave acceptable results.

Elution. — The stained strips are air-dried at room temperature. The dried strips are cut into segments containing albumin, α_1, α_2, β and γ fractions respectively. A blank segment is taken from every strip.

Each segment is weighed and placed in a tube (12 ml) with a ground stopper.

5 ml of 1 N NaOH is added to every tube.

The tube is placed for 20 minutes in a bath with circulating water (70° C). Immediately afterwards *i. e.*, while the tubes are still warm, 5 ml of absolute alcohol is added. The tubes are then shaken vigorously and allowed to cool at room temperature, after which 1 ml of acetic acid is added.

The color, which is read against water in a Beckman photometer at 550 mμ, is stable for several hours.

The extinction value per mg of paper blank is calculated, and the values found for the various fractions corrected accordingly. The distribution of the dye among the fractions is calculated.

Calculation:

Example: albumin fraction

$$\frac{(Ex_{alb.fr.} - \frac{Ex_{blank} \cdot mg_{alb.fr.}}{mg\ blank}) \cdot 100}{\Sigma\, Ex_{fr} - \frac{Ex_{blank}}{mg_{blank}} \cdot \Sigma\, mg_{fr}}$$

Protein-bound hexoses of serum was determined with anthrone according to v. Holt (1954).

EXPERIMENTAL

Identical spots of serum (0.02 ml) are pipetted onto series of moistened filter paper strips. The paper strips were dried at 110° C for 10 minutes. They were then stained according to a modification of Björnesjö's technique, but the concentration of the potassium bisulphite in the sulphite rinse solution ranged from 0.2 to 0.8 per cent. The dye was eluted by the procedure described in the foregoing, but at a number of different temperatures. Each strip included a blank. The extinction values recorded (corrected against the value found for the blank) are given in Fig. 1, from which it is clear that throughout the entire range of temperatures used the values varied with the concentration of the bisulphite and that the absolute values varied with the elution temperature. At room

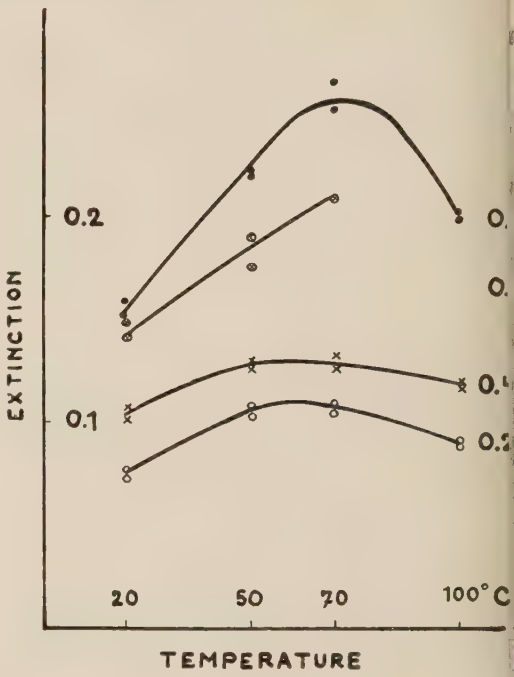
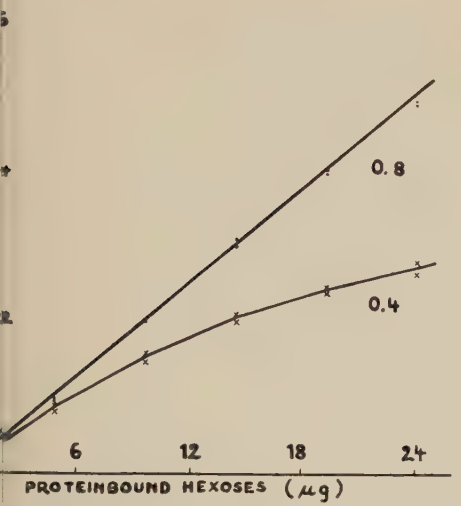


Fig. 1. Influence of temperature and bisulphite concentration in the sulphite rinse solution (range 0.2 to 0.8 g/litre) on the yield obtained on elution of filter paper strips containing stained polysaccharides.

temperature elution of the dye was incomplete. Neither was it complete on prolongation of the elution to 24 hours. The use of a higher temperature accelerated elution, and when elution was carried out within a range of 60° to 80° C, it appeared to be complete within 15 minutes, since the yield did not increase on further prolongation of elution (max. 1 hour). At 100° C the extinction values were lower, and the color of the sample changed from red to red-brown.

The experiments showed: that the use of bisulphite in a concentration higher than that given by Björnesjö increases the intensity



Relationship between the color eluted at and the polysaccharide content of the spots hen using two bisulphite rinse solutions (0.4 and 0.8 g/litre).

olor; that elution at 70° C is more rapid at room temperature; and that boiling the dye.

e purpose of the experiments described was to check whether the amount of luted varied with the quantity of glucoins used.

Dilution series of normal serum was set Two series of spots were stained. One was rinsed with 0.4 per cent bisulphite rinse solution, the other with 0.8 per The results obtained on elution at are given in Fig. 2, from which it is that the concentration of the dye in luate varied almost linearly with the ntration of the glucoproteins in the iments using 0.8 per cent bisulphite, t in those using 0.4 per cent. Variation elution temperature had no effect on this linear relationship. It is true that the curve for the experiments using the higher concentration of bisulphite also showed a tendency to deviate from a straight line, but not along that part corresponding to concentrations of glucoproteins obtainable on electrophoretic separation, where it was practically straight.

The distribution of the glucoproteins among the different electrophoretic fractions was studied by the staining method and elution procedure described in the foregoing. Sera prepared from blood collected from healthy donors in the fasting state was analysed for assessing the normal variation of the relative distribution of the serum fractions. The variation found included not only the biologic spread, but also the error of the method. The relative distribution of the dye among the various electrophoretic fractions was determined (Table I). The hexose content of the various fractions was then calculated from the values found for the neutral protein-bound hexoses (Table II). The protein content of each fraction was also determined (Table III).

Table I. *The distribution per cent of the fuchsin-sulphite stain among the various protein fractions. The figures are calculated from the results obtained on analysis of samples from 34 healthy blood donors. The figures in brackets are taken from Björnesjö's paper (1955).*

	Albumin	α_1	α_2	β	γ
Mean value	8.7 (13.1)	21.2 (17.9)	36.0 (27.9)	25.2 (23.8)	8.2 (17.2)
S. D.	2.1 (4.1)	2.9 (3.8)	3.4 (4.7)	3.5 (3.7)	2.6 (4.5)

Table II. *The amount of protein-bound hexoses in the various electrophoretic fractions given as mg per 100 ml serum. The calculation is based on the assumption that the color varies in direct proportion with the hexose content of the various fractions.*

	Protein-bound hexoses	Albumin	α_1	α_2	β	γ
Mean value	119	10	25	43.5	30	10
S. D.	6.8	2.5	3.5	4.4	5.0	3.2

Table III. *Distribution of the serum proteins among the various electrophoretic fractions. 34 healthy blood donors. All values are given as g per 100 ml serum.*

	Serum protein	Alb.	α_1	α_2	β	γ
Mean	7.1	4.55	0.34	0.53	0.83	0.87
S. D.	0.27	0.18	0.032	0.055	0.11	0.084

Table IV. *Protein and carbohydrate in serum and the electrophoretic γ-fraction from two children with agammaglobulinemia. Serum from patient 1 was collected during an infection.*

Case	g per 100 ml serum		mg per 100 ml serum Protein-bound hexoses in	
	serum protein	γ-globulin	Serum	γ-fraction
1	6.0	0.13	224	1
2	6.5	0.14	141	2

The amount of carbohydrate in the γ-fraction can, theoretically, depend on the amount of carbohydrate bound to the γ-globulin, on the amount of protein-free neutral polysaccharides and on the amount of glucoprotein adsorbed to the paper. In order to elucidat this point, experiments were carried out wit sera from two patients with agammaglobuli nemia. The total content of neutral protein bound hexoses was somewhat higher tha normal in one of the sera because the sampl had been collected when the patient was suf fering from an infection. The results ar given in Table IV. The low carbohydrat content in the fraction of sera from thes patients shows that the normal values give for polysaccharides in the γ-fraction can b ascribed above all to a protein polysaccharid complex with γ-mobility.

DISCUSSION

Adsorption of the glucoproteins to th paper during the electrophoretic migratio involves the risk of the values found for th fastest fractions being fictitiously low. There fore we interrupt the current as soon as th main fractions are distinctly separated.

The stained strips have a relatively intens red background (high blank values). Thi is, however, of minor importance because i can be corrected for by careful gravimetri determination of the mass of the paper be longing to each fraction. The most importan modification of the staining technique is th use of twice the amount of bisulphite in th bisulphite rinse solution. With this increase concentration of the bisulphite the polysac charides are stained more intensely and th amount of dye eluted is directly proportiona to the amount of glucoproteins used. Thi proportionality is not obtained by Björnesjö staining method, even though elution appear to be quantitative.

MAL DISTRIBUTION OF THE POLY-CHARIDES AMONG THE VARIOUS ELECTROPHORETIC FRACTIONS

ble I compares the carbohydrate distri- n found by Björnesjö (1955) for 30 hy adults with that found in the present rial. It is clear that the standard tion for each fraction determined by nesjö's technique is larger than that l for our method. The elution technique appeared to give more reliable values the photometric method. Björnesjö found a relatively larger albumin frac- (13.1 against 8.7) and gamma fraction against 8.2). This might be due to the isulphite concentration with consequent vely more intense staining of fractions a low polysaccharide content. Which e two methods provides the better mir- f the distribution remains to be proved ther methods. Both can be used for al purposes because the absolute value only subordinate importance provided ormal variation is defined. Every ana- must determine the normal variation lf, because slight deviation in the instru- s and technical procedure can result in what different reference variations.

might be mentioned that the major por- of the polysaccharide content given for the γ-fraction consists of the true polysaccharide-protein complex of the same mobility. This is apparent from the results of the analysis of sera from patients with agammaglobulinemia.

SUMMARY

A modification of the method developed by Köiw & Grönwall and by Björnesjö for staining serum polysaccharides after paper electrophoretic separation and a method for eluting the color from the stained papers are described.

The normal range of variation of the glucoprotein pattern is defined.

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Scandinav. J. Clin. & Lab. Investigation 26—29, 8, 1956.

USE OF BRONCHOSPIROMETER FOR THE DETERMINATIO OF THE OXYGEN DEFICIT

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(Received for publication October 12, 1955)

Oxygen deficit (absolute oxygen deficit) is the difference between oxygen uptake, when breathing oxygen, and, on the other hand, ordinary air. The oxygen deficit is determined at rest and at a series of work loads. At each load the subject first works breathing air, then oxygen. If the oxygen uptake when breathing oxygen exceeds that when breathing air by 100 ml per min. or more, the difference is called oxygen deficit; it is considered that this load exceeds the subject's capacity for protracted work. The oxygen deficit has been extensively used as a measure of the functional capacity of respiration and circulation in Germany, Switzerland and France. The value of the method has been recently reviewed by Valentin & Venrath (1952 a, b).

The spirographic oxygen deficit essentially reflects deficient oxygen saturation of arterial blood, though there may also be other contributory factors (Jéquier-Doge, 1950). Technically its determination is simpler than taking arterial blood samples during exercise. Though it is no pure analytical measure, it has the advantage of relating the functional capacity of the respiration and circulation of a subject to his or her exercise tolerance.

The oxygen deficit has generally been d termined by using Knipping's spirograph in which the composition of air may be ke constant, and the subject may rapidly b changed from air to oxygen. As broncho spirometry has lately become widely use and bronchospirographs are available i many places, there is no more need fo special spirographs for the oxygen defic determinations; it may be determined wit any bronchospirograph. The purpose of thi paper is to present the procedure adopte with one type of bronchospirograph; wit small changes the technique is adaptable t other types as well.

METHOD

A "Lundia" bronchospirograph (AB Instru menta, Lund, Sweden) was used. The subject wa first connected to the right spirometer, and th left spirometer was used as a closed oxygen con tainer. The left spirometer was filled with oxyge while the fan mixed it with the "dead space" o the spirometer, and emptied again. After havin flushed the system 5 times it contained 99.5 pe cent oxygen, which is quite sufficient for th accuracy of the method. A weight of approx mately 1 kg was placed on the bell of the spir meter, in order to effect an even flow of oxyg from the container. The error in the volun

ed by the extra weight may easily be cal-ed from the area of the bell, but it is too l essentially to affect the results. The oxygen ining spirometer was connected through thin g and a flow meter with the other spiro-r, which registered the breathing. The oxygen led through the flow meter (range from 0.2 0 l per min.), in order better to control the of oxygen.

he experiment was started by determining the ng oxygen uptake of the subject during 5 tes, breathing air. (Knipping recommends 10 tes.) The volume of the left — oxygen con-ng — spirometer was read immediately before ecting the subject. Thereafter, an amount of en corresponding with the uptake was led the right spirometer, in order to keep the position of air constant. This was done by npting to keep the resting level of breathing horizontal line previously drawn on the kymo-h paper. The volume of the oxygen container e beginning and at the end of the experiment ved the oxygen uptake.

During the determination a pulse count was n; after it, the vital capacity (VC) and the imum breathing capacity (MBC) were deter-ed.

n the exercise tests, the subjects exercised on cycle ergometer, and the loads used were 5, 10 15 kgm meters per sec., according to the in-dual exercise tolerance. The subjects were wed to get adapted to the work during 2 minu-(Knipping 3 min.), and the oxygen uptake was rmined during 3 minutes (Knipping 4 min.). periods were shorter than those used by pping, for patients who had spent long times ped easily got pain in the leg muscles, and efore could not exercise long periods.

While the patient kept working on the bicycle meter, he or she was changed from air to gen, and the oxygen uptake was again deter-ed. For this purpose, the patient was rapidly nected with the left spirometer, the fan of the spirometer stopped. The fan was then again ted, and the oxygen uptake was measured from fall of the resting level, as usually. The pulse was counted during each determination, to-wards the end of it. Immediately after the work the patient was allowed to breath oxygen during 5 minutes, after which the oxygen uptake had practically returned to the resting level, and an eventual oxygen deficit and oxygen debt had been paid off. After adequate rest (from 10 to 15 min.), when the pulse rate had again stabilized, a new determination was made, using the next higher load. Determinations were carried on, according to the fitness of the subject, until the oxygen deficit exceeded 200 ml/min. or the pulse rate approached 150 per min. The accuracy of the method in determining oxygen uptake lies at ± 0.02 l/min.

EXAMPLES OF THE RESULTS

The table shows some examples of the results. The subjects are arranged from best to poorest, according to the oxygen deficit observed with the load of 10 kgm meters per sec. With the load of 5 kgm meters per sec., two of the patients (Ju and Ol) had some oxygen deficit; Ju suffered from cancer of the right lung, Ol was a case of suspected tumor of the left lung.

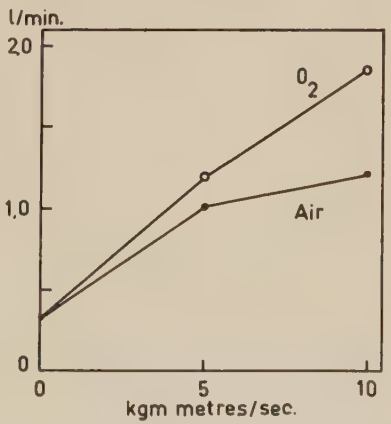

Fig. 1. An example of the results. Oxygen uptake of patient Hä. At the load of 10 kgm meters per sec. the oxygen deficit (= oxygen uptake with O_2 — oxygen uptake with air) is 0.65 liters/min.

Table. *Spiroergometric and pulse data of some subjects with and without respiratory insufficiency.*

Subject	Sex	Age	Exercise 5 kgm meters per sec			Exercise 10 kgm meters per sec			VC in per cent of normal	MBC in per cent of normal	Pulse (in air)		
			O_2-uptake l/min.		O_2-deficit l/min.	O_2-uptake l/min.		O_2-deficit l/min.			Rest	5 kgm/sec	10 kgm/sec
			Air	Oxygen		Air	Oxygen						
Ma	M	26	1.02	1.08	0.06	1.44	1.46	0.02	110	117	72	108	124
Lau	M	31	1.15	1.17	0.02	1.56	1.67	0.11	70	56	116	152	160
Lu	F	43	1.11	1.09	—0.02	1.36	1.59	0.23	93	133	94	138	148
La	M	50	1.17	1.27	0.10	1.51	1.79	0.28	129	79	80	103	112
Ju	M	54	1.10	1.23	0.23	1.25	1.62	0.37	78	49	110	128	150
Ol	M	47	1.12	1.32	0.20	1.39	1.81	0.42	106	114	84	–	160
Hä	M	55	1.02	1.19	0.17	1.21	1.86	0.65	124	90	–	–	–

With the load of 10 kgm meters per sec., five of the patients had a distinct oxygen deficit, from 0.23 to 0.65 liters per min. The first subject was healthy, and he had no significant oxygen deficit even with the load of 15 kgm meters per sec. (0.05 liters per min.). Patient Lau (bronchiectasiae) had a history of severe dypsnoea on exertion, but nevertheless there was no oxygen deficit. Evidently the process had not in this case led to alveolo-respiratory insufficiency and to consequent oxygen deficit; dyspnoea resulted from other factors, primarily from ventilatory insufficiency (low VC and MBC).

Particular attention must be paid to the marked discrepancies between the oxygen deficit and, on the other hand, the VC and the MBC. Similarily, the pulse rate during exercise shows an individual behavior. Thus, these four determinations do not replace each other, but are mutually complementary. This is to be expected, since VC and MBC primarily are measures of ventilatory capacity, whereas oxygen deficit essentially is an indication of alveolo-respiratory insufficiency. Moreover, the results of the spiroergographic examination are affected by cardiac factors; a decompensation of the left heart and consequent pulmonary edema typically cause oxygen deficit during exercise even in patients fully compensated at rest.

CONCLUSIONS

The determination of oxygen deficit gives an objective measure of the functional capacity of the respiration and circulation of a patient. This measure may be of value e. g. in the planning of operative measures in critical cases. It does not replace either ordinary spirometric examination or bronchospirometry. The special value of the method is that the functional capacity of the respiration and circulation of the subject may be quantitatively related to the exercise tolerance. The spiroergometric determination of the oxygen deficit is easily performed with a bronchospirograph, according to the detailed directions given above.

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APPARATUS FOR ANAEROBIC DETERMINATION OF THE p OF BLOOD AT 38 DEGREES CENTIGRADE

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(Received for publication October 20, 1955)

It is a well-known fact that the pH of blood is dependent on the temperature. An increase of between 0.01 and 0.02 pH units is found for each degree centigrade the temperature rises. In the investigations dealing with physicochemical problems concerning the pH and carbon dioxide content (Warburg, 1922; Van Slyke, 1932 and others), the pH was always measured or calculated at a temperature of 38° C. For this and other reasons it is convenient to continue the use of pH values at this temperature.

As the temperature dependence of pH is not constant from one blood sample to another, but varies with the concentration of plasma proteins and especially with the hemoglobin content, it is not advisable, when accurate values are desired, to make the measurements at room temperature and then correct the pH value to 38° C. Instead the measurement proper should be made at 38° C. As it may be difficult to make the measurements under strictly anaerobic conditions, at the same time being sure that the temperature is exactly 38° C., a new apparatus was designed which permits the measurement of the pH in blood under the said conditions with a reproducibility of ± 0.001 pH unit.

METHODS

Description of apparatus

The apparatus, shown in Fig. 1, consists of measuring chamber (1) enveloped by a wat jacket to provide for the maintenance of a co stant temperature by the circulation of therm stated water. A combined glass-calomel electro (2) of standard type, Radiometer combined ele trode, type GK264AT, fits into the measurir chamber. The fluid contact of the calomel ele trode is of the porous disk type.

The apparatus is operated by means of the st cocks (A) and (B) in conjunction with the raisir or lowering of the mercury reservoir (3). The stop cocks shown above the top beaker great facilitate routine work.

To ensure optimal measuring accuracy t apparatus should be used in conjunction with precision pH meter. The results presented he and in the subsequent paper were obtained wi a Radiometer pH-meter type PHM3.

Measuring the pH of blood

Venous blood, the pH of which is to be dete mined, should be withdrawn either without t application of stasis, or with stasis of short dur tion. Syringes moistened with a 5 per cent hepar solution (Heparin NOVO) were employed. Ir mediately after the withdrawal of the blood sar ple the syringe was closed by means of a cannul which was fused together at the tip. Blood m be kept in a tightly closed syringe for 20—30 mi at room temperature without change in pH. Wh

at 0° (ice water) no change in pH is ob-
l for several hours.
e blood may be mixed in the syringe by ad-
g a little mercury in the syringe and rotating
itiously.

aring the apparatus for measurements

e circulation thermostat is started and 10—20
es after the temperature of the circulating
has reached 38° C. (± 0.1°), the electrode
have attained a state of temperature equi-
m.

Calibration of pH Meter

e water is removed from the chamber which
n rinsed twice with standard buffer solution.
r is introduced and temperature equilibrium
ained after 3 minutes. Now the AP control
e pH meter is adjusted to make the meter
the pH of the buffer. As to preparation of
r solution and control of its pH, reference
de to the ensuing paper by Astrup (1955).
e buffer is now removed, the chamber is
l three times with distilled water, the mer-
level is brought to the bottom of the cup,
B is closed and the apparatus is now ready
measurement on a blood sample.

Measurement of pH in blood

short piece of rubber is attached to the tip
e syringe that contains the blood. The rubber
g is pressed against the bottom of the cup.
B is slowly opened, and blood will be drawn
the chamber. When about 1½—2 ml of the
have been admitted cock B is closed and the
ge removed. pH is measured after 4 minutes.

Cleaning the chamber

e mercury level is raised and the blood re-
d by suction. The chamber is rinsed 3—4
with water and a new measurement can now
ade.

Recalibration

may sometimes be necessary to recalibrate
H meter by means of the standard buffer
on.

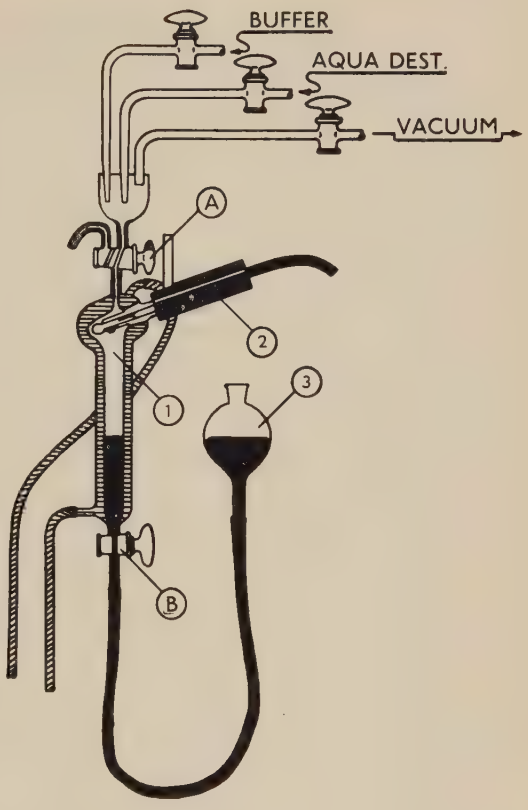

Fig. 1.

Accuracy

Repeated measurements on the same plasma- or blood sample will, provided that they are performed technically correctly, result in differences which do not exceed ± 0.005 pH unit. As the temperature conditions in the electrode are extremely stable, the measuring acuracy is in fact even higher. Thus a variation of only ± 0.001 unit was found in 10 successive measurements on the same plasma.

Checking the calomel electrode

From time to time a check should be made of the potential of the calomel electrode of the combined electrode and of the condition of the fluid

contact. This can be made by means of a separate calomel electrode of the standard type. (A Radiometer calomel electrode type K100, is very suitable for the purpose.)

Firstly the potential between the calomel electrodes is measured, the tip of the separate electrode being brought in contact with the KCl filling of the combined electrode through the side tube. Provided that the separate electrode is known to be in good condition, this potential should not exceed 1—2 mV. Now the measuring chamber is filled with any normal plasma, the stop cock (A) being set to give passage to the beaker which should contain sufficient plasma to allow for the immersion of the tip of the separate calomel electrode Then the potential between the calomel electrodes is determined again. If the condition of the porous disk is satisfactory, this measurement should give the same result as the previous one to within 1 mV. If a greater difference is observed the porous disk should be cleaned. This may be done by immersing the electrode (bulb and disk) in sulfuric acid potassium dichromate.

DISCUSSION

The apparatus described enables pH measurements on blood to be made anaerobically and at a constant temperature of 38° C. If desired, a different temperature can be employed.

Disadvantages encountered when measuring pH in an air thermostat at 38° are avoided. In air it is difficult to maintain a constant temperature during the manipulation and it is especially difficult to maintain a blood temperature of 38° C.

Measurement of pH in blood at 38° C. instead of at room temperature is necessary to obtain accurate determinations, as the factor used to correct the pH actually measured to that prevailing at 38° C. will vary from one blood sample to another.

Graig, Lange, Oberman & Carson (1952) derived the following correction formula:

$$pH_{37^\circ C} = pH_t - 0.0149\ (37 - t)$$

A similar formula was suggested by Rosenthal (1948). However, the constant was found to vary between 0.0103 and 0.0195 in 27 blood samples. The cause of these variations is first of all differences in hemoglobin content, as carbon dioxide is found to diffuse into the blood corpuscles with decreasing temperature, thus causing a rising pH in the plasma. This phenomenon also has the effect that measurement of pH in plasma after the separation of the blood corpuscles will not give reliable information about the pH of the blood sample at 38° C. unless the separation is performed at 38° C. under anaerobic conditions.

SUMMARY

An apparatus for anaerobic measurement of the pH in blood at 38° C. is described. The reproducibility is ± 0.001 pH unit.

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A SIMPLE ELECTROMETRIC TECHNIQUE FOR THE DETERMINATION OF CARBON DIOXIDE TENSION IN BLOOD AND PLASMA, TOTAL CONTENT OF CARBON DIOXIDE IN PLASMA, D BICARBONATE CONTENT IN «SEPARATED» PLASMA AT A FIXED CARBON DIOXIDE TENSION (40 mm Hg)

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(Received for publication October 20, 1955)

he absorption curve for carbon dioxide lood has been the subject of numerous stigations and has formed the basis of umber of papers (for references see rs & Van Slyke, 1932). The curves ribed were plotted by equilibrating d with a gas mixture of known carbon ide tension and then determining the carbon dioxide in blood and plasma. pH could then be calculated according he Henderson-Hasselbalch equation.

this work pH determinations in plasma nown pCO_2 have been used to investi- the absorption of carbon dioxide. A nique is described which is based on stigations of the relation between pH pCO_2 under normal and pathological litions. This technique permits the rometric determination of pCO_2 in ma, the total content of CO_2 in plasma, the bicarbonate concentration in "se- ted" plasma (*i. e.* plasma separated n the cells at the actual pCO_2 of the d) at a pCO_2 of 40 mm Hg.

METHODS

Apparatus

he apparatus used for anaerobic measurement H in blood at 38° C. (Astrup & Schrøder,) was, with few alterations, also used for the tigations described in this paper.

As will be seen from Fig. 1 the chamber is provided with two side tubes, one at the top (a) and the other at the bottom (b). A 2-way stop-cock (C) connects both side tubes with a moistening chamber which contains distilled water enveloped by a water jacket kept at 38° C. A mixture of O_2 and CO_2 passing the moistening chamber is saturated with water vapor at the temperature of the water in the water jacket. From the moistening chamber the gas mixture can be led to the electrode chamber through one of the side tubes, or it can be allowed to escape through cock D when the 2-way cock C is closed. The chamber is provided with an agitator E operated by a coil F which is fed from a pulsator. The agitator moves up and down at a frequency of about 3 cycles per second. The downward movement is brought about by the attraction of the coil and the upward movement by the buoancy.

Measurement of pH

is made fundamentally in the same way as that of the blood (Astrup & Schrøder, 1955).

Equilibration of plasma with oxygen-carbon dioxide mixtures

Equilibration of plasma with mixtures of O_2 and CO_2 of known composition can be made in two different ways. According to the first method the gas mixture enters the measuring chamber through the upper side tube (a) and escapes through cock A. By means of the mercury the plasma level is lowered so much that the agitator

[1] Aided by grants from The States Scientific Foundation.

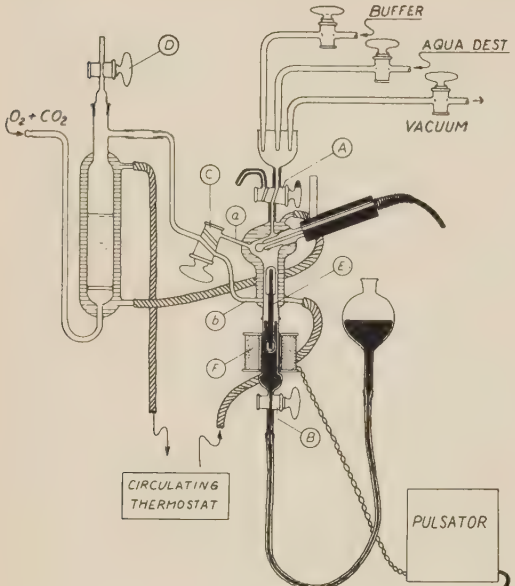

Fig. 1. Arrangement for equilibrating plasma with gas mixtures of known compositions. For detailed explanation see text.

is above the surface of the plasma each time it reaches its top position so that a fresh film of plasma will continuously contact the gas. When the equilibration has been completed (generally in 6—10 minutes), cock B is closed and the plasma level is elevated to surround the electrode. pH is then measured after 2 minutes.

According to the second method the gas mixture enters the electrode chamber through the lower side tube (b), bubbles through the plasma, and escapes through cock A. The plasma level is lowered so that the mercury is immediately below the opening of the gas inlet tube. The plasma surface will then be about 4 cm above the mercury. One drop of octanol is added to avoid foaming. The equilibration takes 4—6 minutes.

The admixture of octanol does not change the pCO_2 at the liquid surface, as the vapor pressure of octanol at 38° C. is only 1 mm Hg. Moreover, octanol does not interfere with the pH measurements. Samples of one plasma equilibrated at the same pCO_2 (led through the upper side tube) hav exactly the same pH (within ± 0.001) whether o not octanol has been added.

Since, however, the bubbles must pass throug a 4 cm layer of fluid, the partial pressure of CO must be increased by ½ to 1 per cent in orde to overcome the hydrostatic pressure exerted b the fluid and this lowers the pH by approximatel 0.003. In the calculations no allowance was mad for this small change.

The O_2—CO_2 mixtures employed and th determination of their pCO_2

Cylinders containing 2, 5 or 10 per cent CO were obtained commercially. The actual CO_2 con tent was determined with a relative accuracy o ± 1 per cent by means of the Haldane apparatus The cylinders were placed in horizontal positio to avoid a decrease in the CO_2 concentration o the outflowing gas during use, as the higher speci fic gravity of CO_2 relative to O_2 causes the CO concentration in the lower part of the cylinder t decrease when standing upright. With the cylinde in horizontal position the percentage of CO_2 doe not change measurably (within ± 1 per cent) i at least 2 months. Thus, 4 analyses made withi 2 months gave the following results: 5.33, 5.2 5.39 and 5.33 per cent CO_2.

The pCO_2 in aqueous solution is calculate from the carbon dioxide concentration measure as follows:

$$pCO_2 = \frac{(B - W) \times \text{per cent } CO_2}{100}$$

where B is the barometric pressure in mm Hg W is the vapor pressure of water at 38° C (= 50 mm Hg).

Measuring pH in a bicarbonate solution o known molarity (M/40 $KHCO_3$) after equilibra tion in the chamber with the gas at 38° C. wa found to be a convenient method of determinin and controlling the CO_2 concentration in the ga mixture.

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[1] I am indebted to the Cardiological Laborator of the University Clinic for these determination

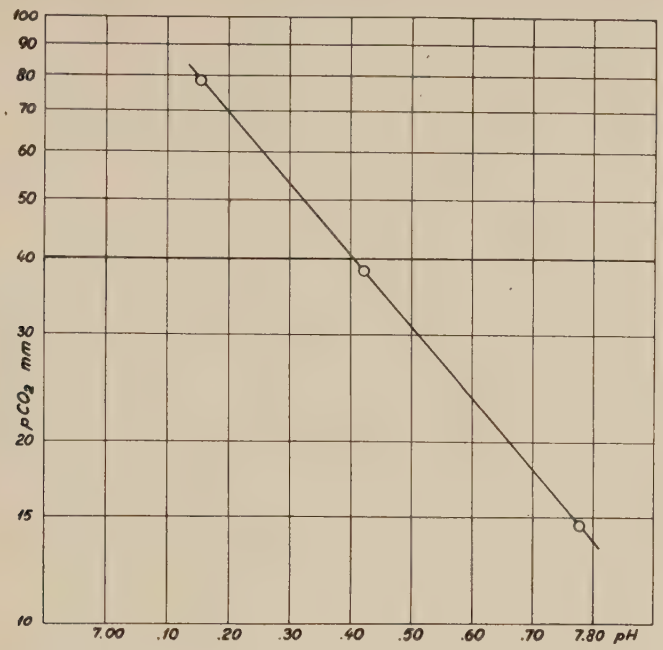


Fig. 2. Relation between pH and pCO_2 at 38° C. in plasma from a normal subject.

ndard buffer solution for adjustment of the pH Meter

ı M/15 phosphate buffer was used, prepared 200 ml M/15 primary potassium phosphate 8 g dry KH_2PO_4 p. a. (Merck) dissolved in r redistilled water) and 800 ml M/15 second- sodium phosphate (11.8667 g $Na_2HPO_4, 2H_2O$ (Merck) dissolved in 1 liter redistilled water). pH of this solution is taken to be 7.360 at C. The variation in pH on repeated prepara- of the buffer solution was within ± 0.001. buffer is best stored in a Pyrex bottle pro- with a sodalime tube.

RESULTS

The relation between pH and pCO_2 of plasma

g. 2 shows a curve of the correlation H and pCO_2 in plasma from a normal an subject. The plotting was made on semilogarithmic paper by means of the pH values measured at 3 different carbon dioxide tensions, *viz.* 14.6, 37.8 and 78.0 mm Hg.

It is apparent that the curve is a straight line. This finding is in agreement with earlier work (*inter al.* Warburg, 1922 and Van Slyke, 1932).

The curve is defined by the pH of the plasma at pCO_2=40 mm Hg and the slope. The pH of the plasma at $pCO_2 = 40$ mm Hg is an expression of the amount of base (OH^-), available for binding CO_2 as HCO_3^-. Twenty-five curves relating pH and pCO_2 in plasma from twenty-five normal subjects were plotted according to the same method. The slopes of the curves were practically identical and there was only a slight parallel displacement between pH 7.36 and 7.41 at $pCO_2 = 40$ mm Hg.

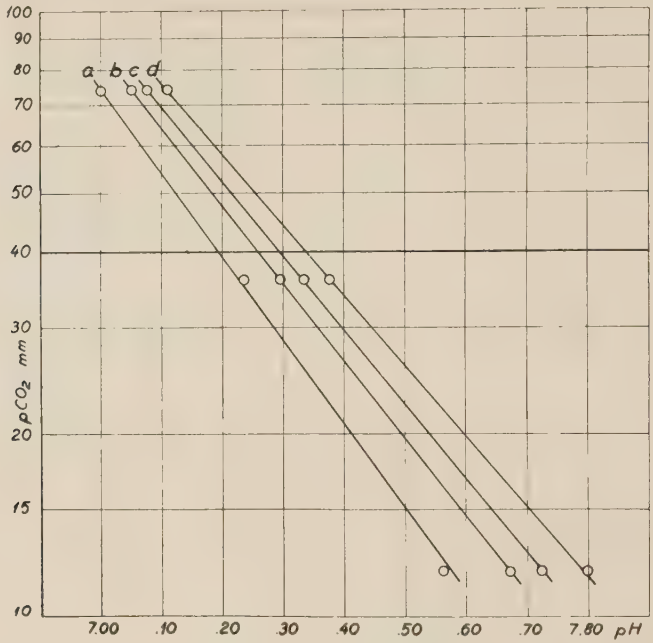


Fig. 3. Relation between pH and pCO_2 at 38° C. in plasma with varying content of protein. Curve a: 13 % protein. Curve b: 11 % protein. Curve c: 9 % protein. Curve d: 7 % protei

The slope of the curve, which is dependent on the buffering action of the plasma, may be influenced by variations in the concentration of protein and base.

The curves in Fig. 3 show the relation between pH and pCO_2 of a normal plasma with a protein concentration of 7.0 per cent when different amounts of human serum albumin are added, resulting in protein concentrations of 9.0, 11.0 and 13.0 per cent. As was to be expected the slope of the curve increases with increasing protein concentration. This effect, however, is so small that variations in plasma protein concentration within the limits 6 to 8 per cent will result in negligable differences in the slope of the curves, provided the concentration of base in the plasma does not change.

The slight displacement to the left of th curves, which is observed on the additio of albumin to normal plasma, is probabl due to the fact that albumin at the pH o the plasma will act as an acid which d creases the base content of the plasma.

Variations in base content will result a steeper curve with decreasing pH. Th will appear from Fig. 4 which illustrat the effect of adding 0.050 ml n HCl an 0.050 ml n NaOH to 2 ml of normal plasm Repeated performances of the experime gave confirmative results. The curves we therefore supposed to correspond to tho of plasma from patients suffering fro metabolic acidosis or alkalosis. In son cases this was verified by experiment Moreover, similar changes in the slope a

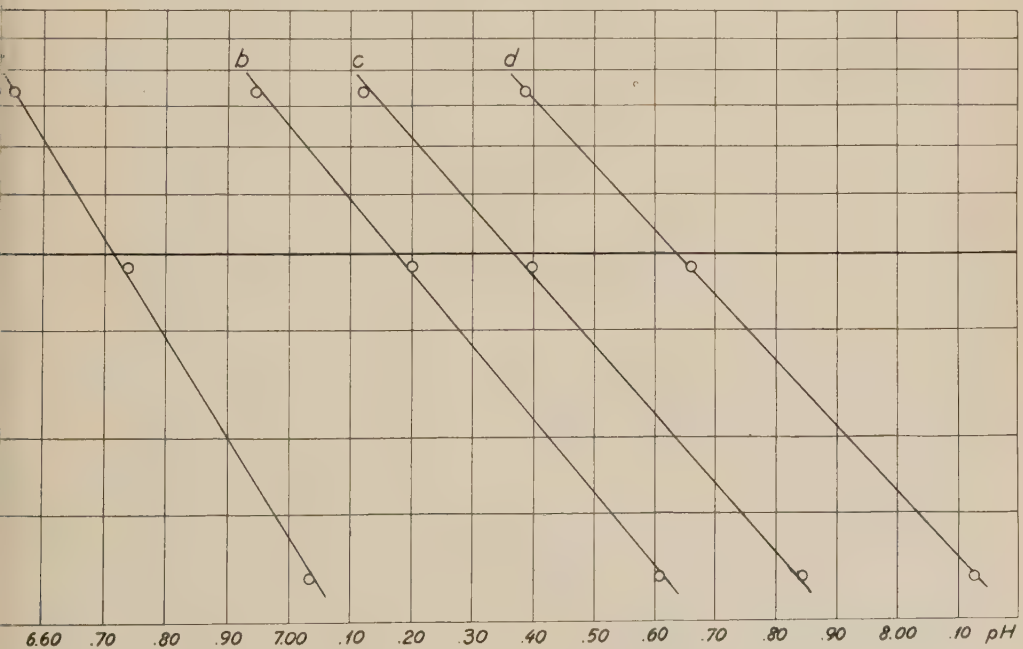
rved in the case of pure bicarbonate phosphate buffer solutions.

may be regarded as an established fact pH—pCO_2 curves of plasma samples normal protein content but with vary- base content will be determined ex- vely by the base content and that the ion and the slope of the curve can be red as soon as one point of the curve is vn. The curve will be approximately llel to the nearest standard curve in the ram of Fig. 5 which was made on the of experiments involving the addition cid and base to normal plasma. Only large pathological changes in the pro- content of plasma will require correc- in the standard diagram. In the event of such changes the simplest method will probably be to determine the pH—pCO_2 curve by measuring the pH after equilibration with two different CO_2 mixtures, containing *e. g.* 5 and 10 per cent of CO_2.

Determining the pCO_2 in plasma

It is evident that the actual pCO_2 in plasma can be determined by means of the actual pH and the pH—pCO_2 curve of the plasma concerned.

After the anaerobic introduction of the sample in the electrode chamber the actual pH is measured. Then the plasma is equilibrated with an O_2—CO_2 mixture of known pCO_2 of approximately 40 mm Hg, the pH is measured again, and the position



g. 3. Relation between pH and pCO_2 at 38° C. in plasma after addition of acid or base.

ırve a: 2 ml plasma+0.050 ml 1 n HCl. Curve c: 2 ml plasma+0.050 ml distilled water.

ırve b: 2 ml plasma+0.025 ml 1 n HCl. Curve d: 2 ml plasma+0.050 ml 1 n NaOH.

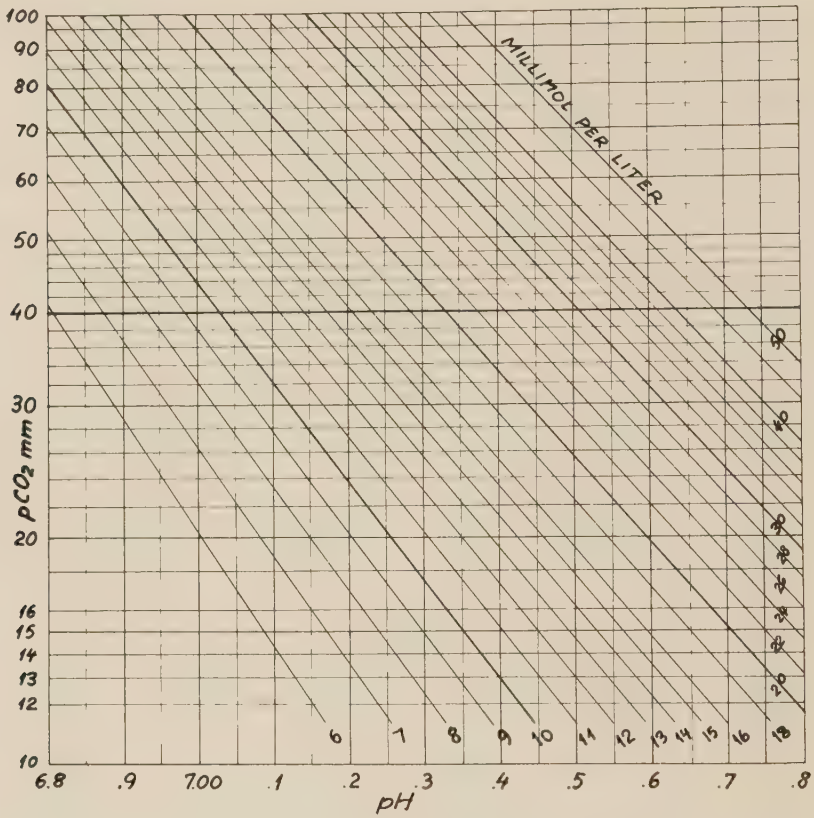


Fig. 5. Diagram relating pH and pCO_2 at varying protein concentration. — The figures on the lines signify bicarbonate concentrations measured in mMol/L at $pCO_2 = 40$ mm Hg.

of the pH—pCO_2 curve is found by using the diagram of Fig. 5. Finally the pCO_2 is determined by means of the curve and the actual pH.

The accuracy of this determination will depend on the accuracy of the pH measurements. When the accuracy of pH is $\pm$ 0.005 pH, the maximum deviation of pCO_2 found will be less than $\pm$ 3 per cent from the true value.

Ten determinations of the actual pH and the pH at pCO_2 = 38.0 mm Hg made on a large sample of plasma showed an even better accuracy, *viz.* $\pm$ 1 per cent.

Determining the total CO_2 in plasma

When the actual pCO_2 and the actual pH of a plasma sample are known, the total content of CO_2 can be calculated by means of the Henderson-Hasselbalch equation:

$$pH = pK_1 + \log \frac{(HCO_3^-)}{(CO_2 + H_2CO_3)}$$

The actual bicarbonate concentration expressed in mMol/L can be calculated by in

ble I. *Determinations of total-CO_2 in plasma samples by electrometric and manometric technique.*

No.	Actual pH	pH at pCO_2 = 40 mm Hg	Bicarbonate content in «separated» plasma at pCO_2 = 40 mm Hg mMol/L	pCO_2 mm Hg	Total-CO_2 calculated mMol/L	Total-CO_2 Van Slyke mMol/L
46	7.024	7.120	12.3	53	14.1	13.5
47	7.363	7.415	24.2	46	26.1	27.8
48	7.317	7.260	16.9	35	18.0	17.2
49	7.288	7.270	17.4	38	18.3	17.9
50	7.182	7.020	9.8	24	9.2	9.7
	7.437	7.440	25.7	40	26.7	26.5
	7.328	7.340	20.4	42	22.1	21.9
	7.392	7.390	22.9	40	24.2	24.1
	7.378	7.442	25.8	46	27.0	27.1
	7.293	7.485	27.8	66	32.2	31.0
	7.350	7.442	25.8	51	28.1	27.7
	7.313	7.400	23.4	51	26.0	26.9
	7.376	7.405	23.7	42	24.9	24.0
	7.470	7.455	26.6	38	27.3	26.8
	7.335	7.270	17.4	34	18.1	18.0
	7.330	7.410	23.9	49	25.9	26.2
	7.330	7.410	23.9	48	25.3	24.8
	7.315	7.379	22.2	48	24.5	24.2
	7.377	7.308	18.9	32	18.7	18.7
	7.235	7.273	17.5	45	19.4	20.5
	7.215	7.348	20.8	57	23.5	24.4
	7.145	7.335	20.2	67	23.8	23.4
	7.299	7.360	21.3	48	23.7	23.3
	7.175	7.350	20.9	64	24.2	24.2
	7.245	7.241	16.2	40	17.6	17.7
	7.370	7.255	16.8	29	16.7	16.9
	7.340	7.228	15.7	29	15.7	16.0
	7.343	7.475	27.8	57	31.0	31.0
	7.374	7.305	18.8	33	19.2	19.1
	7.354	7.426	24.8	49	27.4	27.9
	7.377	7.395	23.2	42	24.6	25.0
	7.366	7.384	22.6	42	24.0	23.6
	7.354	7.390	22.9	43	23.9	23.5
	7.340	7.440	25.7	52	28.1	29.6
	7.334	7.420	24.5	49	26.1	25.3
	7.298	7.410	23.9	54	26.6	26.9
	7.484	7.300	18.6	23	16.4	16.3
	7.266	7.405	23.7	58	26.6	27.7
	7.295	7.412	24.0	55	26.9	27.9
	7.308	7.388	22.8	50	25.2	25.2
	7.360	7.385	22.6	42	23.7	22.8
	7.319	7.380	22.3	47	24.2	25.1
	7.332	7.400	23.4	48	25.5	26.0
	7.288	7.420	24.5	57	27.5	28.1
	7.322	7.410	23.9	50	25.9	25.0
	7.348	7.400	23.4	47	25.8	26.6
	7.324	7.400	23.4	49	25.5	24.2
	7.361	7.423	24.6	47	26.5	27.5
	7.282	7.377	22.1	51	24.3	25.8
	7.313	7.378	22.2	48	24.4	25.8

serting in this equation the pH values measured at 38°, taking pK_1 to be 6.110 and replacing $(CO_2 + H_2CO_3)$, expressed in mMol/L, by pCO_2 in mm Hg multiplied by 0.03. Summing up the bicarbonate concentration and that of the physically dissolved CO_2 gives the total CO_2.

The total CO_2 determined in this way should be identical with the value found by employing the manometric technique, and it should thus be possible to obtain proof of the applicability of the electrometric method. Using the two methods, such determinations were made on a large number of plasma samples (about 90) both from patients and from normal subjects. In Table I the results of determinations on 50 plasma samples are shown. The electrometric determination is made as a single analysis employing the standard diagram shown in Fig. 5. Manometric determinations were made in duplicate by means of Van Slyke's manometric apparatus. The analytical error was $\pm$ 0.3 mMol/L.

The agreement is evidently good as the difference does not exceed $\pm$ 1.0 mMol/L in about 90 per cent of the samples. In all samples the difference was less than $\pm$ 1.7 mMol/L.

Determining the bicarbonate content of "separated" plasma at $pCO_2 = 40$ mm Hg

On the basis of the pH—pCO_2 curve it is possible to find the pH which the plasma (separated at the actual pCO_2) will have at $pCO_2 = 40$ mm Hg. When the value is inserted in the Henderson-Hasselbalch equation, it is a simple matter to calculate the bicarbonate concentration at $pCO_2 = 40$ mm Hg.

If an O_2—CO_2 mixture with a pCO_2 of about 40 mm Hg is used for the equilibration, variations, if any, in the standard curve will be without significance for the determination of pH at pCO_2=40 mm Hg. The result will thus depend on a single pH measurement. If it is accurate to ± 0.005 pH, the determination of bicarbonate content at $pCO_2 = 40$ mm Hg will be accurate to ± 2 per cent. This accuracy was confirmed by several analyses of samples from one plasma.

Normal values of pH, pCO_2 and bicarbonate content at pCO_2=40 mm Hg in "separated" plasma from venous blood

Normal values were determined in 23 men and women aged 19—39 years. Blood from the cubital vein was withdrawn after stasis of short duration. The blood samples were taken between 9 a.m. and 3 p.m. The results appear in Table II which includes information on the hemoglobin concentration, the oxygen saturation, the plasma protein concentration, and the content of total CO_2 in plasma determined by means of Van Slyke's manometric apparatus.

The pH was found to vary between 7.282 and 7.350, and the pCO_2 between 46 and 58 mm Hg. The bicarbonate content plasma at $pCO_2 = 40$ mm Hg was fou to be between 21.3 and 25.7 mMol/L.

DISCUSSION

Plasma is easily equilibrated with C by bubbling the gas through the plasma w addition of octanol to avoid foaming. Eq libration of whole blood, however, offe difficulties because of its tendency to hem lyze. This holds true, no matter whether t O_2—CO_2 mixture is admitted above t surface of the blood or it is bubbled throu the blood. In the former case the hemoly will be caused by the mechanical action the magnetic agitator used to accelerate t equilibration. In the latter case the sm addition of octanol required to prevent t blood from foaming will cause the blood hemolyze very rapidly. Incidentally th phenomenon can be utilized to determi the pH in blood corpuscles. The prelimina results have shown that the pH of hem lyzed blood at $pCO_2 = 40$ mm Hg fro normal subjects is constantly about 0.12 0.14 pH units below that of the plasma $pCO_2 = 40$ mm Hg.

The fixation of the numerical value pH in the buffer solution was the subje of careful considerations and investigatio The pH of the buffer solution is given 7.35 at 38° C. (Consolazio, Johnson Marek, 1951). When measuring with hydrogen electrode, we found the pH to 7.37 at 38° C. When employing a pK_1 val of 6.110 in the Henderson-Hasselbal equation (Hastings, Sendroy & Van Slyk 1928 and Dill, Daly & Forbes, 1937), found by examining 70 samples that t pH of the buffer solution should be tak

le II. *Normal values for pH, pCO_2 and bicarbonate content at pCO_2=40 mm Hg in separated plasma form veneous blood.*

Plasma-protein g/100 ml	Hemo-globin g/100 ml	O_2-saturation per cent	pH in blood at 38°	Actual pCO_2 mm Hg	pH in »separated« plasma at pCO_2= 40 mm Hg	Bicarbonate content in »separated« plasma at pCO_2=40mm Hg mMol/L	Total-CO_2 Van Slyke mMol/L
7.2	17.1	50	7.311	51	7.40	23.4	24.5
7.5	14.0	55	7.350	47	7.40	23.4	26.6
7.4	12.7	61	7.340	48	7.41	23.9	25.0
7.4	14.6	49	7.327	52	7.42	24.5	28.1
7.4	12.9	47	7.344	46	7.40	23.4	26.0
7.2	12.1	36	7.319	47	7.38	22.3	25.1
7.4	13.5	44	7.317	47	7.39	22.9	22.8
7.3	13.9	33	7.332	47	7.39	22.9	25.2
7.5	18.0	39	7.326	51	7.41	23.9	27.9
7.4	13.3	31	7.278	57	7.41	23.9	27.7
7.4	14.3	30	7.322	51	7.41	23.9	26.9
7.4	14.4	47	7.350	47	7.42	24.5	25.3
7.5	16.1	30	7.342	58	7.44	25.7	29.6
7.5	13.6		7.321	45	7.37	21.8	23.3
7.5	14.4		7.329	53	7.42	24.5	27.8
7.1	12.9		7.315	48	7.38	22.3	24.2
7.3	12.9		7.313	51	7.40	23.4	26.9
7.4	13.9		7.313	56	7.38	22.3	25.8
7.2	14.8		7.315	46	7.36	21.3	24.4
7.5	13.9		7.307	53	7.41	23.9	27.7
7.8	14.6		7.334	52	7.42	24.5	27.5
7.0	13.3		7.282	51	7.38	22.3	25.8
7.5	14.7		7.320	50	7.40	23.4	25.9

: 7.360, and the pH meter adjusted
dingly.

e method used for determining the
in plasma can also be used for deter-
g the pCO_2 in whole blood, when the
pH of the whole blood at 38° C. is
yed together with a pH—pCO_2 curve
ing to the plasma of the same blood.
this connection the question arose
er it is permissible to use plasma
ed through centrifugation at room
rature instead of at 38° C. This was
igated by using blood from patients
ng a high sedimentation rate. The
was allowed to separate anaerobically at 38° C., 22° C. and 0° C. The separation was completed within 15 minutes. Tightly stoppered tubes filled completely with blood were used. The pH was measured at 38° C. both in whole blood and in the three plasma samples, and the corresponding pH—pCO_2 curves were made. Table III shows the results of an experiment.

Table III.

Plasma separated at	Actual pH	Actual pCO_2	pH at pCO_2 =38.0 mm	Total-CO_2 mMol/L Van Slyke
38° C	7.398	38	7.402	26.5
22° C	7.453	34	7.402	26.0
0° C	7.507	30	7.407	25.5

It is evident from the table that the pH in plasma increases with decreasing separation temperature and that the pCO_2 decreases. The pCO_2 curves, on the other hand, are nearly identical in the 3 plasma samples. Moreover, a decrease in total CO_2 is found. Quite similar results were found in another experiment. Thus it makes no difference in the pH—pCO_2 curve in plasma whether the separation of blood corpuscles is made at 38° C. or 0° C. However, if the actual pCO_2 of whole blood is desired, pH must be measured in whole blood or plasma separated at 38° C. A more detailed discussion of the problems concerning the absorption of carbon dioxide in blood corpuscles at different temperatures will be postponed to a subsequent paper.

As will be seen from Table I, satisfactory agreement is found between total CO_2 measured by electrometric and manometric technique when the pH of the reference buffer is taken to be 7.360 and the pK_1 6.110.

Theoretically a better agreement than that actually found was not to be expected as the calculation of total CO_2 from the pH values measured must depend not only on the accuracy of these measurements, but also on variations in pK_1 from one plasma to another. In the literature pK_1 is stated to vary between 6.09 and 6.13 and to be 6.11 on the average (Hastings, Sendroy & Van Slyke, 1928). Consider a plasma sample with an actual pH of 7.40 and an actual pCO_2 of 50 mm Hg. If a pK_1 of 6.09, 6.11 or 6.13 is chosen, the corresponding values of the total CO_2 will be 32,1, 30.7 and 29.4 mMol/L, respectively.

The agreement of the values of the to CO_2 found by means of the two methods an indication of the accuracy and reliabil of the electrometric method for the det mination in both normal and pathologi plasma samples of the total CO_2 and es cially of pCO_2.

The determination of the bicarbon content of "separated" plasma at pCO_2 40 mm Hg is very simple and lends its advantageously to serial analyses. In th analyses the gas mixture is bubbled throu plasma samples in open tubes and the p is mesaured immediately after the equ bration.

The values of the bicarbonate content "true plasma" separated from the cells $pCO_2 = 40$ mm Hg and at 38° C. can found in a similar way by using pH—pC curves for whole blood. This technique w be described in a subsequent paper.

The normal values found of pH a pCO_2 in blood from the arm vein are good agreement with previously publish normal values. By means of a less accur technique somewhat higher values (betwe 7.34 and 7.43) were found of the pH venous blood (Astrup, 1954) and simila somewhat lower values of pCO_2 (betwe 40 and 53 mm Hg). This difference m be due to the fact that the pH of venc blood is dependent on the technique e ployed for venipuncture, which may ha involved different duration of the stasis the two series of investigations. Furth more, the temperature was not exac 38° C. in the earlier investigations.

SUMMARY

simple electrometric technique is de-
ed which permits the determination of
in plasma, the total content of CO_2
asma, and the bicarbonate content of
rated" plasma at $pCO_2 = 40$ mm Hg.
method is based exclusively on pH
urements and on the laws governing
elation between the pH and the pCO_2
asma.

od agreement between the results ob-
l by means of the electrometric and
metric methods were found in 90
es from different subjects.

e determination of the actual pCO_2
he bicarbonate content of "separated"
a at $pCO_2 = 40$ mm Hg is possible
an accuracy better than ± 3 per cent.
rmal values are given for the pH,
and the bicarbonate content in "se-
ed" plasma at $pCO_2 = 40$ mm Hg in
s blood.

ACKNOWLEDGMENT

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TOTAL EXCHANGEABLE POTASSIUM IN NORMAL SUBJECTS WITH SPECIAL REFERENCE TO CHANGES WITH AGE

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Measurements of the total potassium pool of the body have attracted growing attention during recent years, since the importance of potassium loss has been established in a number of conditions, and since it has become evident that only limited conclusions as regards intracellular potassium concentration can be drawn from measurements of extracellular potassium concentration.

With the exception of the method based upon measurements of gamma emission from the naturally occurring potassium isotope K^{40} (Burch & Spiers, 1954; Rundo & Sagild, 1955; Miller & Marinelli, 1955), the only procedure available for estimating the total body content of potassium in living subjects is the injection of the potassium isotope K^{42}. After equilibration of injected potassium with body potassium available for exchange, the specific activity of the urine can be used as a measure of the exchangeable potassium (K^e) by the application of the dilution principle.

Since the introduction of this method in clinical medicine (Corsa, Olney, Steenburg, Ball & Moore, 1950) normal values in a group of young males (Corsa *et al.*, 1950) and young females (Aikawa, Harrell & Eisenberg, 1952) have been reported, bu apparently the possible changes with ag have not been taken into consideration.

The purpose of this communication is t report the results of measurements of K in normal individuals of both sexes through out the adult life span and briefly to discus the implications of the findings.

MATERIAL AND METHODS

Material

The subjects studied were 44 male and 3 female volunteers. 20 males and 12 females be longed to the age group 18—30 years, and in eac of the groups 31—60 years and 61—80 years ther were 12 males and 12 females.

The young age group was made up of health medical students, hospital employees and friend the middle group also contained the above, bu was supplemented by 10 hospitalized subjects wit conditions in which potassium abnormalities wer highly improbable. These subjects and the diag noses have been indicated in the tables. The ol age group was made up of inhabitants of "D gamles by" (municipal homes for aged).[1] Som of the members of the latter group were marke by arteriosclerotic changes, but were include since these changes are so frequent as almost t constitute a "normal" finding.

[1] Through the courtesy of Dr. T. Geill.

Methods

ıe potassium isotope K^{42} was procured from er, Norway. Each shipment contained 50 ml sterile 3.75 per cent solution of potassium ide in water with a K^{42} activity ranging from to 280 microcuries per ml in different ship- s at the time of arrival. The material was d to be free from any significant radioactive mination, especially with sodium. 10 ml of sterile solution was diluted to 500 ml with e isotonic saline.

rom this diluted solution each subject received ly 20 ml intravenously, containing 0.2 meq of sium. No side effects from the injections noted.

he injections were given 2 to 16 hours after rrival of the isotope and the dose of radio- ty varied from 30 to 60 microcuries of K^{42}. microcuries of K^{42} deliver about 80 millirep. e tissue in a 70 kg person (the half-life of sotope is 12.4 hours). Since the maximal per- ble dose for a week is 300 millirep., the dose was only about a fourth of the maximal ssible dose.

or the injection the system proposed by Bar- & Fellers (1947) was used. Repeated checks ed that the error from the injection only ded upon the accuracy with which the pipet- was performed, and that this error could be ed to less than $^1/_2$ per cent.

fore the injection the subjects were required id, and their height and weight were obtained. They were not necessarily fasting and continued their normal activities. All urine was collected and pooled until the morning 24 to 36 hours after the injection. Subsequently two "spot" urines were obtained, before the subjects received breakfast (at least 20 ml each).

In a few instances the individual samples of urine obtained throughout the experiment were analysed. Measurements of specific activity in these samples confirmed the finding of Corsa *et al.*, that equilibrium between injected K^{42} and body potassium is obtained within 12 to 20 hours.

Measurements of radioactivity

The activity of the urine specimens was determined with a fluid counter (M 6, the "Veall"-type). All counting rates were at least 500 to 3.000 counts per minute, with a background of 10—12 counts per minute. At the range of counting rate, no correction for dead time was necessary. The measurements of the urines were compared to standard curves, prepared from measurements of appropriate dilutions (usually 500 fold) of the solution, used for the injections. Duplicate measurements of specimens from the pooled urine and from each of the "spot"-urines were made. All measurements were expressed in per cent of the dose given.

Potassium concentration in the "spot"-urines were determined with a flame photometer with internal standard, constructed in the laboratory.

Calculations

cording to the dilution formula

Total radioactive K in the body (in per cent of dose given) / Total exchangeable K (in meq)

s

activity in "spot"-urine (in per cent of dose given) / Potassium content in "spot"-urine (in meq)

equilibrium is obtained. Therefore

(in meq) = K^{42} in the body (in %) × F

e F denotes potassium content in a given "spot"-urine volume (in meq) divided by the radioactivity content in the same volume (in per cent of dose given). All measurements of activity and chemical potassium in the "spot"-urines were reported for a volume of 10 ml.

Table I. *Exchangeable potassium content of men, 18—30 years.*

Subject	Age yrs.	K^e meq	K^e/kg	K^e/cm	K^e/m^2
1	24	3670	53.9	21.3	2060
2	29	4150	50.3	22.2	2020
3	21	4450	59.7	24.6	2300
4	28	3650	50.3	20.4	1920
5	30	2800	46.2	16.1	1630
6	24	3380	46.8	18.9	1780
7	23	4620	60.7	24.3	2300
8	26	3530	44.2	20.4	1840
9	19	3650	48.0	20.1	1870
10	20	3900	53.3	21.4	2030
11	20	4650	59.5	27.0	2450
12	30	2900	42.0	17.0	1620
13	29	3040	46.4	17.3	1700
14	21	2640	39.7	14.8	1450
15	21	3640	47.6	20.8	1900
16	23	2260	38.2	13.2	1350
17	18	2500	50.0	15.2	1630
18	27	3270	51.6	19.4	1910
19	23	3330	42.0	18.1	1670
20	18	4860	55.8	24.9	2210
Mean	23.7	3545	49.3	19.9	1880
S.D.		740	6.43	3.6	290
Corr. coeff.			0.81	0.66	0.79

Table II. *Exchangeable potassium content of men, 31—60 years.*

Subject	Age yrs.	Diagnosis	K^e meq	K^e/kg	K^e/cm	K^e/m^2
1	31	skin rash	3980	48.5	22.9	2020
2	39	br. asthma	3060	48.3	18.7	1810
3	31	neurosis	3160	46.5	18.8	1790
4	47	normal	3420	43.8	19.8	1800
5	54	normal	3040	46.1	17.7	1710
6	52	br. asthma	3620	49.9	20.6	1930
7	58	normal	3040	43.4	17.0	1630
8	59	normal	3730	42.6	22.5	1900
9	44	br. asthma	3060	50.6	17.1	1740
10	60	coron scle.	2640	44.9	16.3	1630
11	33	normal	2990	49.4	17.7	1770
12	56	normal	3660	40.4	19.9	1710
Mean	47.0		3283	46.2	19.1	1786
S.D.			389	3.1	2.1	118
Corr. coeff.				0.87	0.38	0.84

Table III. *Exchangeable potassium conte of men, 61—80 years.*

Subject	Age yrs.	K^e meq	K^e/kg	K^e/cm	K^e/r
1	71	2450	35.0	16.0	147(
2	73	2740	40.0	16.9	158(
3	73	2420	38.1	14.8	143(
4	71	2900	34.4	16.7	147(
5	67	2850	36.1	17.3	153(
6	71	2540	37.4	15.7	148(
7	72	2960	36.3	17.3	154(
8	73	2720	34.9	15.5	142(
9	66	2810	37.8	16.7	153(
10	69	2330	35.4	14.4	137(
11	70	2340	37.3	14.6	142(
12	75	2540	33.4	14.8	135(
Mean	70.9	2633	36.3	15.9	146
S.D.		224	2.5	1.1	71
Corr. coeff.			0.95	0.62	0.82

Table IV. *Exchangeable potassium conte of women, 18—30 years.*

Subject	Age yrs.	K^e meq	K^e/kg	K^e/cm	K^e/m
1	18	2790	46.5	16.7	167(
2	18	2520	37.2	15.7	147(
3	22	2230	39.8	14.0	143(
4	20	2230	43.8	14.0	149(
5	18	2920	33.4	16.4	142(
6	18	2220	42.3	13.5	142(
7	22	2280	38.6	13.7	137(
8	20	2410	43.0	15.0	153(
9	25	2980	38.2	17.7	159(
10	23	2850	40.7	17.2	160
11	30	2360	40.3	14.8	148(
12	28	2700	36.0	16.5	148(
Mean	21.8	2540	40.0	15.4	149(
S.D.		290	3.3	1.5	9(
Corr. coeff.			0.86	0.70	0.86

We have disregarded the loss of radioactivi in feces and perspiration, which has been fou insignificant by others (Blainey, Cooke, Quint & Scott, 1954), and calculated the K^{42} remaini in the body at the time of the voiding of t "spot"-urines as 100 minus K^{42} excreted in t pooled urine (in per cent).

ble V. *Exchangeable potassium content of women, 31—60 years.*

Age yrs.	Diagnosis	K^e meq	K^e/kg	K^e/cm	K^e/m²
58	normal	2200	24.6	14.0	1290
37	normal	2540	39.0	15.4	1490
41	cardiac. neurosis	2550	32.0	15.3	1360
54	normal	2390	31.4	15.0	1340
47	resp. neurosis	2430	38.0	15.0	1440
36	normal	1990	36.8	12.8	1310
49	neurosis	1730	32.3	11.2	1150
42	hyst. aphonia	2110	40.0	13.3	1390
33	normal	2020	44.9	13.0	1430
34	normal	1900	42.8	11.9	1340
37	normal	2370	37.9	14.4	1410
52	normal	2390	34.6	14.1	1330
n 43.3		2218	36.2	13.8	1356
		267	5.3	1.3	100
. coeff.			0.79	0.84	0.87

he term F, which expresses how many milli-valents of potassium will be excreted in the t"-urine with one per cent of the K^{42} given, been found rather convenient. It gives a rough nate of total exchangeable potassium, when ious urinary loss of K^{42} is unknown and is pendent of decay or amount of activity given.

Experimental error

he difference between the F-values obtained wo subsequent "spot"-urines, expressed as per of the mean value, varied between 0 and 8.7 cent, with a mean of 4.2 per cent. This agrees y well with the reproducibility of the method: ix subjects included in this series, the dif-nce between duplicate determinations varied een 60 and 270 milliequivalents. For the further ussion of the errors involved in this method, reader is referred to the paper of Blainey ., 1954. It seems to be agreed generally, that difference between two measurements in the subject should exceed 250 milliequivalents e considered significant.

Table VI. *Exchangeable potassium content of women, 61—80 years.*

Subject	Age yrs.	K^e meq	K^e/kg	K^e/cm	K^e/m²
1	63	2120	34.8	12.9	1280
2	73	1850	27.9	10.9	1050
3	69	1640	21.1	10.3	910
4	77	1970	35.9	12.2	1250
5	65	1700	22.6	10.6	940
6	80	1500	25.3	9.2	920
7	73	1510	25.0	10.1	970
8	78	1560	31.7	10.3	1080
9	69	2230	29.0	14.6	1280
10	64	2180	24.9	12.9	1100
11	72	2300	29.0	13.7	1220
12	70	1750	27.9	11.4	1090
Mean	71.1	1859	27.9	11.6	1090
S.D.		292	4.5	1.7	138
Corr. coeff.			0.54	0.48	0.58

Table VII. *Regression lines of K^e on body weight.*

Group	Sex	Age yrs.	Regression line	S.D.
I	M	18—30	K^e(meq) = 68.6 Wt (kg) — 1350	446
II	M	31—60	K^e(meq) = 31.6 Wt (kg) + 1022	202
III	M	61—80	K^e(meq) = 26.7 Wt (kg) + 689	120
IV	F	18—30	K^e(meq) = 21.9 Wt (kg) + 1134	157
V	F	31—60	K^e(meq) = 18.3 Wt (kg) + 1095	171
VI	F	61—80	K^e(meq) = 13.5 Wt (kg) + 510	261

RESULTS

The results of 80 measurements of K^e are tabulated according to sex and age in Tables I—VI, and the relation between weight and K^e is graphically represented in Fig. 1.

Inspection of correlation coefficients between K^e and weight, height and surface area, respectively, shows that the best overall correlation was observed between K^e and weight. The regression lines for these two variables were calculated in all groups

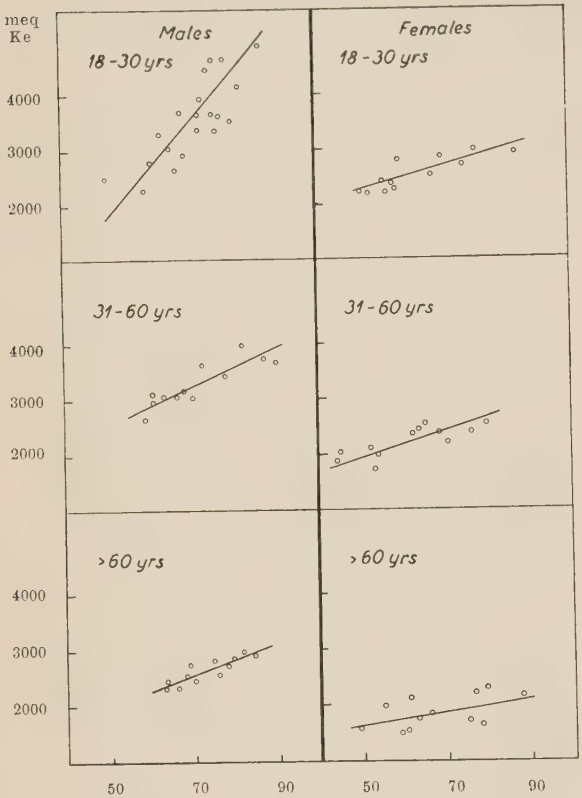

Fig. 1. Relation between kilo body weight and Ke in 80 normal adult subjects.

and presented in Table VII. It appears that the increase in K^e with increasing weight is greatest in the young group of both sexes and becomes successively smaller with increasing age. It appears further, that the increase with weight is smaller in the female than in the male groups.

The mean values of K^e and K^e per kilo body weight are higher in the young groups of both sexes and decrease with increasing age (Tables I—VI). Between groups II—III, IV—V and V—VI the difference in mean values of K^e/kg was statistically significant at the 5 per cent level, but n between groups I—II.

Mean values of K^e are higher in mal than in females.

DISCUSSION

The results of measurements of K^e young males, presented in Table I agr well with figures reported by Corsa *et a* for a similar group, while those for female in the same age group (Table IV) a somewhat higher than the figures reporte by Aikawa *et al.* for a comparable grou No measurements in groups of older peop have been reported previously.

Although the average height and weigh in the six groups reported do not quite re flect trends in the general population, it i believed that the groups are comparable a far as general living conditions and stat of health and nutrition are concerned. Th decrease in K^e per kilo body weight wit increasing age in both sexes is therefor assumed to represent a consequence of th ageing process itself. This decrease might b explained on the basis of different assump tions:

Increase in absolute fat content of the body with age

Although systematic quantitative da covering the whole life span are not avai able, the general trend of fat in man is we known; the peaks of fatness being in ear childhood and in late maturity (Keys Brožek, 1953). This is documented b measurements of body water (Prentic Siri, Berlin, Hyde, Parsons, Joiner & Law rence, 1952; Edelman, Olney, James, Brook & Moore, 1952), skin fold thickness a

ific gravity (Brožek, 1952). Since fatty e is essentially potassium free, this e would cause a decrease of K^e per unit ht with age, but K^e would remain un- ed. In the present study an absolute ease of K^e as well as a decrease per weight was demonstrated. Granted pos- deficiencies in the sampling of the ects, it is still difficult to explain why older subjects should show such uni- ly lower K^e-values, if the decrease only relative to the increased fat con- Furthermore, the mean increase in ht in the older groups was not con- able in this material.

crease in relative fat content of the body with age

here is considerable evidence to suggest the gross body changes in weight with are of a complex nature, and that in- ents in fatty tissue occur simultaneously decrements in other tissues. The well wn decrease in basal metabolism with can be accounted for by this decrement, "disuse atrophy" of the muscles, which ain some 70 per cent of body potassium aracteristic of normal ageing, (Roessle Roulet, 1932).

he decrease in exchangeable potassium age demonstrated in this paper is ably best explained on the basis of the mption of an absolute and relative in- se in fat combined with an absolute and ive decrease in lean body mass.

he sex differences can be interpreted similar fashion: the "physiological ess" and relatively smaller fraction ean tissue in women — a corrolary which is the lower basal metabolism referred to body weight — is well established. It should be noted that the sex difference in K^e becomes smaller with age.

From a theoretical point of view two more mechanisms might explain the changes of K^e with age:

Decrease of cellular concentration of potassium with age

In a variety of clinical disorders associated with exogenous potassium loss, cellular potassium has been shown to be exchanged by sodium and thus cellular concentration of potassium decreased. Whether a similar process might be associated with ageing is unknown, and the data presented allow no interpretation in this respect.

Relative increase in non-exchanged fraction of body potassium with age

Due to the relatively short half-life of the isotope K^{42} (12.4 hours), observations with safe tracer doses can only be extended to 40—60 hours. It is known that within this space of time complete exchange with total body potassium will not be obtained. The non-exchanged fraction has been estimated to 5—10 per cent 20 hours after the injection (Corsa *et al.* 1950; Rundo & Sagild, 1955). It might be postulated that the exchange process is slower in older individuals and the estimated exchangeable potassium content after 24—36 hours therefore lower. However, this assumption could hardly explain the magnitude of the difference in mean K^e between the young and old groups, which in men weighing 70 kilos constitutes some 500—1000 meq of potassium. Furthermore, unpublished experiments in our laboratory with observations

extended over 60 hours have given no reason to assume that the exchange process should be slower in older individuals.

The interpretation of data for exchangeable potassium in pathological states has been somewhat hampered by the fact that normal values, whether stated in terms of total K^e or referred to weight, height or surface units, have shown wide variations. It is believed that a closer interpretation is possible, if the naturally occurring changes with age are taken into account.

SUMMARY

1. Measurements of exchangeable potassium in 80 normal adult subjects of both sexes are reported.

2. Total exchangeable potassium (K^e) and K^e per kilo body weight decreases with age in both sexes.

3. Total exchangeable potassium is lower in women than in men.

4. It is suggested that the decrease of K^e and K^e per kilo body weight with age is caused by the absolute and relative increase in fat together with an absolute and relative decrease in lean body mass.

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ME URIC ACID VALUES IN NORMAL HUMAN SUBJECTS

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ost of the previous investigations on acid in the human organism were based nspecific analytical methods. Kalckar 7) showed that specific measurement ric acid is possible by photometric de- nation of the extinction decrease after ion of uricase. Prætorius (1949) pro- d a highly purified uricase preparation, by making a specific enzymatic uric determination practicable. By the pre- titrimetric and colorimetric methods chner-Mortensen, Folin) the results of neasurements were influenced in vary- degrees and directions by other sub- es present in urine and especially in na.

ie publication of Prætorius' method fies renewed investigations into the al values for uric acid in the organism. have been the object of the present .

MATERIAL AND METHODS

e subjects were hospitalized patients with no olic or renal diseases, who may therefore in onnection be regarded as normals. They were a low-purine diet for at least 24 hours be- fore the experiment was commenced. The blood samples were drawn a few hours after breakfast.

Th uric acid concntrations in plasma and urine were determined by Prætorius' method (1949), later improved by Prætorius & Poulsen (1953).

The uric acid concentrations in plasma and urine clearance technique (Smith, Goldring & Chasis, 1938), in some cases without catheter à demeure and bladder washing. In these latter cases the urine volumes discharged were 5 to 10 ml/min. or more. The clearances were estimated as the average of at least three successive collecting periods of 15 to 30 minutes each. The inulin concentration was about 25 to 50 mg/100 ml, being kept at a constant level by continuous intravenous infusion. Inulin was analysed according to Alving, Rubin & Miller's method (1939) in Brun's (1946) or Bojesen's (1952) modification, which gave the same results.

The difference between duplicate analyses did not exceed 3—4 per cent.

RESULTS

The plasma uric acid concentration and the 24-hour uric acid excretion were determined in 53 men and 18 women. The individual results are averages of 3—4 days' successive determinations. The values are seen in Table I.

Table I. *Plasma concentration and excretion of uric acid in normals on low-purine diet.*

Number examined	Average observation period (days)	Average plasma concentration of uric acid (mg/100 ml)	Average excretion of uric acid (mg/ 24 hours)
Men 53	3.8	4.4±0.4*)	401±42
Women 18	3.7	4.1±0.5*)	321±34

*) Standard deviation.

Table II. *Plasma concentration and 24-hour excretion of uric acid in normals.*

Investigator	Number		Plasma concentration of uric acid (mg/100 ml)	24 hour excretion of uric acid (mg)	Method for uric acid determination
Brøchner-Mortensen (1940)	20	♂	7.6	374	Brøchner-Mortensen
		♀	6.4		
Friedman & Byers (1950)	6		—	390	Folin
Gutman & Yü (1952)	13	♂	5.4	416	Buchanan *et al.*
		♀	4.1		
Hasner et al. (1952)	84		4.4*)	—	Prætorius
Lous & Syl-vest (1954)	166	♂	5.4*)	—	Prætorius
	201	♀	3.8—4.8*)		
Gjørup, Poulsen & Prætorius (1955)	143	♂	5.04±1.0*)**)	—	Prætorius
	157	♀	3.84±0.7*)**)		

*) On ordinary diet.
**) Standard deviation.

Table II shows the results of other investigations into normal uric acid values. As might be expected, our figures are slight lower than those found by other investig tors, on the basis of Prætorius' method, subjects on an ordinary diet.

Table III. *Corrected uric acid clearan and ratio of uric acid clearance to inu clearance in 21 normal subjects.*

Experiment	Sex	Cluric acid (ml/min.)	Cluric acid corr. to 1.73 m² (ml/min.)	Clinulin ml/min.)	Cluric a / Clinuli (per ce
1	m	10.3	9.6	100	10.6
2	m	6.7	5.7	106	6.3
3	m	12.3	10.4	108	11.3
4	m	9.7	8.7	128	7.6
5	m	15.5	14.8	127	12.2
6	m	6.3	5.7	108	5.8
7	f	13.0	13.5	76	17.1
8	f	11.8	12.1	97	12.2
9	m	13.2	12.2	112	11.8
10	f	10.9	12.7	94	11.6
11	m	11.8	10.2	139	8.5
12	m	8.7	8.3	125	7.0
13	f	11.4	11.4	87	13.1
14	f	10.0	11.5	61	16.4
15	m	8.2	7.9	134	6.1
16	f	10.7	12.4	68	15.7
17	f	11.1	12.7	92	12.1
18	f	9.5	9.8	126	7.5
19	m	7.4	6.5	130	5.7
20	m	6.9	7.2	97	7.1
21	m	7.0	6.6	126	5.1
Average			10.0		10.0
S. D.			1.1		1.4

The uric acid clearance was determin in 21 subjects. Simultaneous estimation the glomerular filtration rate, measured the inulin clearance, made it possible calculate the ratio of uric acid clearance inulin clearance, which indicates the e cretion percentage of uric acid, on the a sumption of complete filtration of uric ac The results are set out in Table III.

e IV. *Previous investigations into the cid clearance and the ratio of uric acid clearance to inulin clearance.*

gator	Number of determinations	Cluric acid (ml/min.)	Cluric acid / Clinulin (per cent)
ıer-Mortensen 0)	16	6.93	—
s et al. 0)	8	11.5	10.2
t 3)	3	12.0	10.2
s et al. 4)	?	15.4 corr.	—
r et al. 0)	2	16.4	13.2
& McCaw 1)	?	15.0	8—10
Work	21	10.0 corr.	10.0

ble IV compares our results with those ed by other workers. None of their s were obtained by enzymatic methods.

SUMMARY

ne normal figures are given for uric in the human organism. The deter- ions were based on a specific enzy- method (Prætorius). On a low-purine ne plasma uric acid concentration was to be 4.4 mg/100 ml and 4.1 mg/- ml in 53 men and 18 women. The ge 24-hour excretion was 401 mg and ng. The uric acid clearance (corrected 3 m^2 of surface) was estimated in 21 and found to average 10.0 ml/min. ratio of uric acid clearance to inulin nce was 10.0 per cent.

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ERMINATION OF 17- HYDROXYCORTICOSTERONE IN IPHERAL PLASMA FROM DOGS AND HUMANS WITH RADIOACTIVE P- IODOPHENYLSULFONYLIC ACID ANHYDRIDE (PIPSAN)

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INTRODUCTION

ntitative analysis of corticosteroids sma is the tool necessary to obtain information about the role played adrenals in regulation of the intact and the human being under normal thological conditions. In any method uantitative determination of corti- ids, two difficulties must be over- *viz.* the low concentration of hormone t in plasma under normal conditions e presence of many closely related unds in plasma. Marrian (1955) arizes the present situation by saying: andard deviation of ± 10 % at op- steroid concentrations is the best that n reasonably hope for with most of ethods in use at present time; and some methods particularly at sub- l steroid concentrations one may o be content with a S.D. of ± 25— As to the recovery his opinion is 5 per cent recovery from plasma of pure steroid will be very satisfactory. ble I are summarized some of the ls published. The specificity is de- t upon chromatographic separation on paper or on columns and on a al reaction, flourescence or absorb- light in the u—v range of the puri- fied substance. The method devised for determination of groups of corticosteroids such as the method of Nelson & Samuels (1952) and that of Kassenaar, Molenaar, Nijland & Querido (1955) have been used extensively to obtain some information about changes in the concentration under experimental or clinical conditions. The data obtained must be evaluated with caution because these methods do not offer any routine criterion of the specificity of the method which may be applied under all conditions. In the methods published by Morris & Williams (1953 & 1955) and of Sweat, Abbott, Jeffries & Bliss (1953) the chromatographic technique offers better possibilities for separation of the different steroids, however the final measurement add very little to the specificity of the whole procedure. It may well be that these methods really are specific to the extent claimed, but some reservation seems reasonable until more crucial experiments have been carried out to confirm this point. This criticism is sustained by the discrepancy between some of the results obtained by these methods and by theoretically more specific methods. The method of Avidi, Simpson, Tait & Whitehead (1954) has shown a much lower concentration of 17-

Table I. *Some characteristics of the methods for determination of corticosteroids in blood.*

Author	Isolation	Final determination	Specificity	Range	Recovery in per cent	Accuracy
Nelson and Samuels (1952)	column chromatography	the reaction of Porter—Silber	probably only comp. F	0.5=10 μg (10 ml plasma)	80—130	
Bush (1952)	paper chromatography	density and area of spots	comp. F, B and E separately	5—20 μg	?	semi-quantitative
Morris and Williams (1953)	column chromatography	polarography	comp. F, B and E separately	(20 ml plasma)	80—90	
Sweat *et al.* (1953)	chromatography	fluorescence	separately comp. B and F	(5 ml plasma)	?	?
Avivi *et al.* (1954)	chromatography on column	determination of $_{1}^{4}C$ and $_{3}H$ activity	Comp. F high specificity	about 1 μg	about 70	±25 per c (st. error)
Kassenaar *et al.* (1955)	liquid/liquid partition	the reaction of Porter—Silber	probably only comp F	(20 ml plasma)	80—104	deviation on double determinations ab 1 μg/100 m plasma
Morris and Williams (1955)	gradient elution of column chromatography	U—V absorbtion reduction of blue tetrazoliumchloride	comp. F, B and E separately	(40 ml plasma)	80—90	
The present	paper chromatography of derivatives	^{35}S ^{131}J	comp F	0.2—10 μg (5 ml plasma)	80—90	S.d. in the ra 0.2—10 μg: ±6 per cent

hydroxycorticosterone in human peripheral plasma than the other methods (2.5 ± 0.5 μg/100 ml whole blood) and the findings of Bush & Sandberg (1953) that human peripheral plasma contained very little corticosterone in comparison with 17-hydroxycorticosterone are in disagreement with the data of Morris & Williams (1953) and Sweat *et al.* (1953). It seems obvious that if the determination of these hormones in plasma shall be of any conclusive value, a method must be available which makes possible to control the specificity in ea individual determination. Otherwise p sible changes in the concentration of me bolites of steroids and other interferi substances under the experimental con tions will mask the changes in the conc tration of the substance looked for.

The analytical principle of isotopic de vation developed primarily for amino ac by Keston, Udenfriend & Levy (1950)

tentatively applied to this analytical em by Avidi *et al.* (1954). They used : acid anhydricde labelled with ^{3}H and is the reagents in the determination of rocortin (aldosterone) in plasma. They asized the great theoretical advantage ved in this procedure, but for reasons h will be discussed later they did not ed in developing an applicable method. i *et al.* (1954) obtained better results attempt to determine the level of 17- oxycorticostereone in plasma according e other principle of double tracer tech- introduced for amino acids by Keston ospaluto (1952). Avidi *et al.* (1954) ^{14}C labelled 17-hydroxycorticosterone ^{3}H labelled acetic acid anhydride. The ficity was high and the recovery was lent as far as can be estimated from ather low accuracy. The sensitivity of type of technique is limited by the fic activity of the ^{14}C labelled steroid, he difficulty of obtaining the different of labelled steroids makes the method acticable for the general determination rticosteroids.

e present paper deals with a method upon the same principle of Keston (1950) but using another reagent. The iple is in fact merely a variation of the ic dilution principle and can very y be described as follows: The un- n amount of steroid to be measured terified (together with other reactive ounds present) with a substance label- vith a radioactive isotope (^{35}S). Pro- the esterification of that steroid is itative, the amount of activity present e ester will be a measure of the un- n amount of steroid present. Direct advantage can however not be taken of this fact, in measuring the unknown amount, since when only small amounts of the ester are present it cannot be isolated in uncontaminated state, either quantitatively, or with a reproducible recovery. The losses which inevitably occur during purification can, however, be indicated by adding a known amount of the steroidester labelled with another isotope (^{131} I), the activity of which can be measured independently of that of the first (^{35}S). From the ratio between the two activities in a sample of steroid-ester isolated and the known amount of the ^{131}I-labelled ester added, the unknown amount of steroid originally present can then be calculated. From the discussion below it will appear that the double tracer technique has some additional advantages which make the whole procedure feasible as a routine method.

Bojesen, Keston & Carsiotis (1953) showed that corticosteroids such as cortisone, hydrocortisone and desoxycorticosterone were easily esterified with p-iodophenylsulfonylic acid anhydride (pipsan). Only one of the hydroxyl groups of hydrocortisone was esterified and the yield of the ester was about 70 per cent. Studies of this esterification with the aim of developing a satisfactory analysis have resulted in a procedure yielding quantitative esterification of pure hydrocortisone. Based upon this procedure a method for specific determination of hydrocortisone in plasma from dogs and humans has been worked out. The overall recovery is about 86 per cent and the standard deviation $\pm$ 6 per cent at the level of 0.2—1.3 μg preformed in plasma samples from dogs and humans.

METHODS

Reagents

Extraction Procedure

Chloroform (Riedel de Haën: Für Analyse Spezialqualität Seruminstitut)
Methanol (p. a. Merck)
n-Hexane (M & B B. P. 67—69)
Toluene (p. a. Merck)
Ethyl acetate (Merck)
Anhydrous sodium sulfate.

Esterification

Chloroform purified, anhydrous: The chloroform (the above mentioned quality) is washed three times with 1/3 the volume of sulfuric acid, then with about 1/3 the volume of water and dried with sodium sulfate. Finally it is destilled over BaO under anhydrous conditions at 62 degrees (760 mm). Only the complete anhydrous fraction is collected. As long as it still contains a little water the chloroform will turn opalescent when cooled to —5 to —10 degrees. This purified and dry chloroform is very unstable and must be used immediately after destillation.

Pyridine, purified, anhydrous: Pyridine (p. a. Baker) is refluxed and destilled over BaO the day it is used. Only the fraction boiling at 115.7 degrees (760 mm) is collected under anhydrous conditions.

Pipsan with ^{35}S (Pipsan ^{35}S) was prepared from pipsyl chloride (Keston *et al.* 1950) according to the method used by Bojesen *et al.* 1953. The overall yield of activity as Pipsan ^{35}S was about 30 per cent. The anhydride is a white powder with a sharp melting point 220—221 degrees. It must be stored over P_2O_5 at —10 to —20 degrees, otherwise the melting point will drop indicating some breakdown and it will absorb water which eventually will ruin the esterification.

Indicator: Of each steroid to be analysed the pipsyl ester labelled with ^{131}I is prepared as follows: About 5 mg steroid is dissolved in 0.3 ml 7 per cent pyridine in chloroform (both purified as described above). 30 mg "Pipsan ^{131}I" prepared from pipsyl chloride ^{131}I (Keston *et al.* 194 with about 1 m Curie ^{131}I is added slowly (2— minutes) under vigorous shaking, then 0.3 m 7 per cent pyridine in chloroform is added and few minutes later the chloroform is evaporate in a stream of nitrogen. The sticky residue stirred into 2 ml benzene, centrifuged and the clea benzene solution is pipetted to another tube which the ester is precipitated with n-pentane. Th solvent is removed and the residue dried and di solved in a few drops of methanol from whic the ester is precipitated with 3—4 drops of wate Following centrifugation (3200 rpm. in an o dinary Ecco-Superior-M centrifuge) for half a hour the methanol — water is removed and th precipitate dried in a stream of nitrogen. Thes two precipitations are repeated once. The ester finally dissolved in benzene in which it is store at room temp. When 20—30 analyses are per formed per week this solution will suffice fo about 6 weeks. The purity can be checked b chromatography and radioauthography of th chromatogram.

Substance of reference of "Pipsan ^{35}S" (pre paration of pipsyl glycine ^{35}S): 200—300 m glycine ethyl hydrochloride is dissolved in 5 ml o 4 N NH_4OH and extracted twice with ether. Th ether is dried with sodium sulfate and evaporate The residue is dissolved in 0.5 ml 7 per cent pyri dine in chloroform (both purified and anhydrous and about 5 mg "pipsan ^{35}S" is added. Chloroform and pyridine are evaporated in a stream of nitro gen and the ester is hydrolysed with 4—5 drop of 0.5 N NaOH by shaking the tube in the vibra tor and keeping the tube dipped into a boilin waterbath. When acidified the pipsyl glycine wi precipitate. The precipitate is spun down and th supernatant is removed. The precipitate is the shaken up in ether and transferred to another tub Ammonia saturated ether is added and the am monium salt of pipsyl-glycine ester falls ou These two precipitations are repeated twic Finally the acid is dissolved in a few ml water an the concentration is determined by means of th ultraviolet absorption. The solution is diluted wit 0.2 N HCl and the extinction coefficient at 250 μ is determined in a Beckman apparatus. The mola extinction coefficient is 15900.

Apparatus and Glassware

s anhydrous conditions are necessary in order btain complete esterification, the glass tubes in this part of the procedure were heated in ream of dry nitrogen. 5 mm × 80 mm tubes fitted with a rubber cover in which two in- ns are made, one for a pasteur pipette for of the nitrogen (or for the Carlsberg pipet- and one for a very small and narrow glass for outlet of nitrogen (or equilibration of pressure). The reagents (anhydride and pyri-) are added with Carlsberg pipettes which just been dried by heating in a stream of gen and they are operated with a screw on sed rubber tube,

vibrator A. C. 220 volt (Resiprotor, piston , Denmark) is used for shaking the tubes. romatography: Glass tanks (20×24×52 cm) sed. A fan with a diameter of 8 cm driven motor outside the tank (1500 r.p.m.) is used crease the speed with which equilibrium is ned. The tank, solvents and separating fun- used are placed in a constant temp. room at C. Three sides of the tank are covered with paper, one of which dips into a tray placed e bottom containing the methanol-water phase the two others reach the bottom of the tank are soaked with the organic phase. The cover ne tank has a hole through which the moving e can be added to the tray carrying the paper s when equilibrium is completed. The filter r used for chromatography was Whatman 1 washed with 2 N acetic acid and water, and n strips 2 cm wide, one strip being used for analysis.

olvent systems: A: 70 per cent methanol- r and 40 per cent toluene-ligroin.

: 90 per cent methanol-water and 40 per cent ne-ligroine. The ligroine was distilled at 75° C.

adioautography: X-Ray film (Kodak, Blue d, Code 3, 24 × 30 cm²).

ounting technique: Tracerlab's Utility Scaler a G—M end window tube TGC—2. Window ness 1.9 mg/cm² C. The sample is placed on luminum planchet. The counting chamber of is supplied with a shutter of aluminum foil mm in a frame. This device a determination of the activity of the sample with or without a filter between the sample and the G—M tube without any change in the position of the sample.

Analytical procedure

1) *Extraction of Comp. F. (17-hydroxycorticosterone) from plasma*

10 ml or 5 ml plasma were beaten up in 15 ml chloroform (stabilized with 1 per cent methanol). The chloroform layer is siphoned to another flask with a small depression in the bottom for destillation *in vacuo*. This destillation is carried out with a water suction pump at 40—50° C., and a slow stream of nitrogen is passed through a fine capillary into the fluid. When about one ml is left the remnant is transferred to a small glass tube (7 × 100 mm). The flask is washed twice with 0.5 ml of methanol and twice with 0.5 ml of chloroform. The sample is taken down to dryness by passing a stream of dry nitrogen over it. The residue is dissolved in one ml of 70 per cent methanol in water. This solution is washed twice with 0.5 ml of 20 per cent toluene in n-hexane, and the methanol-water is evaporated with the nitrogen. The residue is now taken up into one ml ethyl acetate and washed twice with 0.5 ml water. The ethyl acetate is dried with 50—100 mg sodium sulfate and transferred quantitatively to another tube. The ethyl acetate is evaporated in a stream of nitrogen and the residue is finally transferred quantitatively to the reaction tube with dry purified chloroform. This tube was just previously dried as described above. The chloroform is evaporated with dry nitrogen.

2) *Esterification*

The residue is dissolved in 25 μl 7 per cent purified pyridine in dry chloroform and the tube is placed in the vibrator dipping into a Dewar flask with ethylene glycol at 0° C. The vibrator is started and three mg "pipsan ^{35}S" dissolved in 100 μl dry pure chloroform is added rapidly (about 10 seconds) with a dry Carlsberg pipette. Immediately an additional 25 μl 7 per cent pyridine in chloroform is added. The tube is now removed from the vibrator and placed in a rack. After not more than 5 minutes, it is again placed

in the vibrator, the rubber cover is removed, a small drop of water added and then after carefully shaking the indicator is added as soon as possible.

3) *Addition of the indicator*

The indicator is kept in stock dissolved in benzene. As the activity of the iodine decays rather rapidly the volume containing the required activity for one analysis rises rapidly. Since the procedures involve partition between the organic phase and a water phase it is necessary to add the indicator in pure chloroform in a fixed volume (0.05 ml) of pure chloroform. The solution of the indicator in chloroform is made each day and adjusted so that 0.05 ml contains 600—800 counts/min. The activity should not be less, because the following isolation procedure involves loss of about 50 per cent to 70 per cent, and not more because then the S/I ratio per mol of steroid present in the analysis will be difficult to determine with accuracy. For the purpose of calibration of the group of analyses made with one particular solution of indicator, 0.05 ml is transferred to two empty tubes before the indicator is added to any tube containing sulfur activity (this order of operation prevents any contamination of these calibration samples). After this step in the procedure it is not necessary to operate in a quantitative or reproducible way because the indicator will account for losses, however no unnecessary loss must be tolerated because low counting rates in the final determination of S/I ratio will lower the accuracy of the analysis.

4) *Isolation of the ester*

In the esterification procedure the "Pipsan ^{35}S" is added in very large excess relative to the amount of ester formed, but most of it can easily be removed since the pyridinium salt of the p-iodophenylsulfonylic acid is very soluble in water. The chloroform phase is washed twice with water, and once with 50 per cent methanol in water, dried in a stream of nitrogen and transferred to the paper strips with 10 per cent methanol in chloroform. Two variations of the system proposed by Bush (1952) were found to give excellent chromatograms of the esters of corticosterone, 17-hydroxycorticosterone and cortisone. The paper strips are placed in the first tank (A), the fan is started and the moving phase is run into the tray containing the strips. When the solvent front has reached the starting line (15 minutes) the fan is stopped. 45 minutes later the chromatograms are taken out and the spot with iodine activity is localised with a G-M tube supplied with a cover of aluminum foil. The spot is chopped in small pieces and the ester is eluted by extraction four times with 0.5 ml of 10 per cent methanol in chloroform. The extraction fluid is evaporated with nitrogen and the residue is transferred to another paper and a chromatogram is run in the second tank (B) for three hours after an equilibrium period of one hour with the fan on. The chromatograms are radioautographed overnight. The next day the spot containing the indicator is eluted as described above and if a check for purity of the spot is desired it is devided into a lower and upper half, which are eludated separately.

5) *Counting and Calculation*

The planchet is counted in the same position without and with the aluminum foil between the planchet and the counter. In this way values for the total activity ($\bar{s}$) and for the high energy radiation ($\bar{c}$) are obtained. From these values the S/I ratio of the sample is calculated by correcting for the fraction of iodine activity (f_I) and the fraction of sulfur activity (f_S) which passes the filter. For a given filter f_I and f_S are constant and are determined separately. The formula is

$$\bar{s} = S + I \text{ and } \bar{c} = f_I + f_S$$

from which

$$\frac{S}{I} = \frac{f_I \bar{s} - \bar{c}}{\bar{c} - f_S \bar{s}}$$

In the method here described the indicator is used not only to account for any loss in the isolation procedure and error in the geometry of the counting procedure, but also to calibrate the analysis without any determination of specific activity. Only S/I ratios enter the calculation.

For the purpose of calibration, the S/I ratio of a sample is determined containing a known number of moles of the "pipsan ^{35}S" and the same

nt of the indicator solution with the ^{131}I ed ester as used in the analysis. Since pipsyl- e is easy to prepare it has been used as the nce and the concentration of the solution is nined spectrometricaly by use of the extinc- oefficient of the substance. A certain num- f moles, enough to give a convenient S/I with the indicator activity used, is added to vo standard tubes. A few drops of acetic re added and the tube is dried in a stream rogen. A small drop of acetic acid and a rops of 10 per cent methanol in chloroform sed for the transfer of the homogeneous n to the planchet. The S/I ratio of this et is then counted with the same counter as known sample. The moles unknown (mol_x) sample are then calculated from the for- where mol_{st} means the number of moles syl-glycine ^{35}S added to the standard.

$$mol_x = \frac{(S/I)_x}{(S/I)_{st.}} \cdot mol_{st.}$$

theory the ester of the steroid would be a correct reference substance to assure that dine and sulfur activity were distributed in lar way on the planchet. However the error determination of $(S/I)_{st}$ was not greater he counting error and therefore this simpler ure was adopted.

RESULTS AND DISCUSSION

Esterification

nen a blank analysis is run in parallel tubes containing known amounts of 17-hydroxycorticosterone, the spots iodine activity in the blank have shown negligible sulfur activity in compari- vith the S/I ratios found in the sam- given in Table II. This indicates first l that the ester is sufficiently stable the conditions chosen so that the activity in the indicator does not ex- e with the sulfur activity in the rea- and that the system used is efficient in ating the excess of the reagent from ster.

Table II. *S/I ratio and recovery of pure 17-hydroxycorticosterone in four different eperiments.*

μg comp. F	S/I ratio	Calculated recovery in per cent	
1.32	2.87—2.90—2.90—2.94	97	98
		98	99
0.66	1.35—1.51	95	105
0.66	2.72—2.86	105	108
0.575	2.43—2.54	103	107

It is apparent from the results given in Table II that the esterification is quantitative and that none of the iodine activity in the indicator is exchanged with the sulfur activity. It has been considered important to prove this point as convincingly as possible. Therefore the esterification was carried out with Comp. F. and "pipsan ^{35}S" and a certain large amount of ^{131}I labelled ester of comp. B. (corticosterone) was added. In the final chromatogram no iodine activity in the spot of the ester of comp. F was found and no sulfur activity in the spot of the ester of comp. B. The Rf of the two esters were 0.5 (comp. F) and 0.8 (comp. B) in the system of tank A.

It has not been possible so far to establish clearcut limits of the different conditions employed in the esterification procedure. However, it can within a reasonable degree of certainty be stated that the conditions chosen can not be subject to much variation. The only condition which makes the procedure a little difficult is the demand of anhydrous conditions. There is no doubt that the esterification sometimes has been complete even when this condition was not fullfilled, but it has not been possible, in spite of many attempts, to obtain a reliable pro-

cedure unless every trace of water was removed from the reaction tube. The "pipsan ^{35}S" must have the proper melting point. When it is hydrolysed the acid formed will always take some water as it is a very hygroscopic substance. The amount of the reagent (3 mg) chosen will saturate 0.1 ml chloroform. Unfortunately chloroform seems to be the only one of the common solvents in which "pipsan" is soluble to any considerable extent and it has been found that 2 mg "pipsan" will not yield 100 per cent esterification. The amount of pyridine is a slight excess relative to the amount necessary to neutralize the acid formed, and the experiments have shown that the yield is not optimal when it is not in excess. It made no difference whether this excess was two or threefold but it is to be expected that a large excess will reduce the yield because Reichstein & Fuchs (1940) did find that a large excess of pyridine will split the analogous ester of "tosyl." Furtherore a large excess of pyridine would be inconvenient in the purification procedure because it would keep some "pipsyl acid" in the organic phase when partitioned with water. A low temperature was chosen because it was reasonable to expect that this would improve the esterification and one experiment showed that this actually was the case when conditions were not completely anhydrous. The yield then was 97 per cent 0° C., 84 per cent at room temp. and 80 per cent at 40° C. The esterification is complete within a couple of minutes and it is to be expected, and was also found experimentally, that the indicator must be added as soon as possible after completion of the reaction especially when water is added. The water is added to be sure that no anhydride is left when th indicator is added in order to prevent a re-esterification of the indicator ester, any of it is hydrolyzed.

Isolation procedure

The large excess of "pipsan" is remov by washing with water and as only a fe per cent of the ester formed and added w be lost in washing with 50 per cent meth nol/water this step has been introduced remove other impurities. The procedu with two paper chromatograms has bee chosen as the most convenient method obtain the pure ester of comp. F. When th analysis is run with pure comp. F. simp partition procedures followed by one chr matography in the system of tank A wi most often give a pure spot. It has howev not been possible to achieve this consistentl and the procedure with two chromatogra was therefore chosen as the most reliabl The R_f's of the esters in question are co venient. In the system of tank A Comp. E and F will run with R_f's at 0.7—0.8, 0 and 0.4—0.5 respectively. In the system tank B the R_f of the ester of Comp. F about 0.3 and therefore this chromatogra is allowed to overrun. The radioautograph is necessary to localize the spots accurate The positions are too different on stri run in parallel to rely on tracing with monitor, and the amount of material is n large enough to allow a localisation in ultr violet light.

Counting procedure

The minimal theoretical error of the me thod arises from the counting procedur The two counting rates entering the ca

ion ($\bar{s}$ and $\bar{c}$) of the S/I ratio have determined with a theoretical s. d. of per cent in all the data given in this r. In Fig. 1 is given the theoretical s. d. /I = R for two different values of $\bar{c}$ function of the R-value itself. hen $\bar{c}$ has the value of about 100 c/min. heoretical s. d. of the determination of is about 4 per cent, when the back- nd is about 25 c/min. and the total per counted is of the magnitude of . With the same background and total per counted the s. d. of $\bar{c}$ will be 1.5 ent. The s. d. of $\bar{s}$ is always 1—1.5 ent. In this calculation the two filter rs (f_f and f_s) are supposed to be 0.6 0.03 respectively. The figure shows that $\bar{c}$ is below four times the background high values of R do not give satis- ry results. On the other hand higher s of $\bar{c}$ than eight to ten times the back- nd do not lower the s. d. of the deter- tion of R appreciably. This means that certain sulfur activity in the sample ptimal value of $\bar{c}$ is about 6—10 times background. The amount of indicator l must therefore be adjusted so that actually will be the case when a loss in solation procedure of about 50 per cent nsidered. The curves in Fig. 1 can be to find out whether the counting tech- used is optimal because the s. d. of ted determination of R of the same het must be as indicated in the figure e technique is sufficiently good. From s. d. of the final determination of the it must be concluded that it will be nable to work with an accuracy of 3—5 per cent in the chemical part of nalysis.

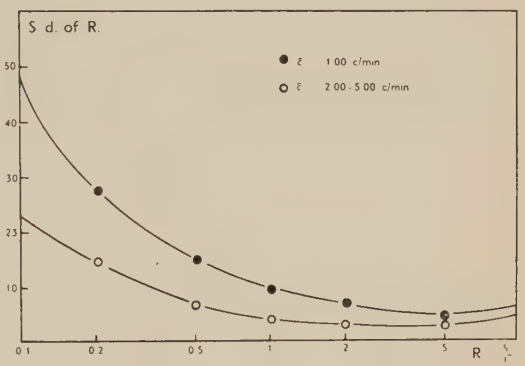

Fig. 1. The s. d. of S/I = R as a function of the magnitude of R. This function for two values of $\bar{c}$ (100 and 200—500 c/min.) when the background is 25 c/min. and the total number of counts is about 5000. The values are calculated from the formula $R = \frac{f_1 \bar{s} - \bar{c}}{\bar{c} - f_s \bar{s}}$ and f_1 and f_s: 0.6 and 0.03.

Extraction of plasma

The method of Kassenaar *et al.* (1955) has been followed to some extent. Table III shows that the extraction yields a residue from which comp. F can be recovered almost quantitatively as the ester.

Table III. *Data on the percentage recovery of comp. F added to plasma extract of dogs and humans. The amount of plasma extracted was 10 ml for dogs and 5 ml for humans.*

	μg Comp. F added	μg Comp. F preformed	Percentage recovery	
	1.15	0.25	93	
dogs	0.66	0.59	95	98
	0.58	0.30	96	
	0.58	1.21	120	92
	0.58	0.50	93	98
humans	0.58	0.51	88	103
	0.55	0.65	91	99
	0.30	0.30	100	100
	0.45	0.50	92	102

Table IV. *Check on the chromatographic purity of the ester of preformed comp. F.*

	Plasma ml	µg Comp. F	S/I ratios upper half	S/I ratios lower half
dogs	10	0.24	0.435	0.405
	10	0.25	0.45	0.47
	10	0.59	1.58	1.72
	10	0.59	1.74	1.52
humans	10	1.17	2.76	2.68
	10	1.26	2.95	2.88
	5	0.88	3.35	2.85
	5	0.88	3.48	2.95
	5	0.51	0.92	0.92
	5	0.49	0.89	1.00
	5	0.53	1:02	0.85
	5	0.49	0.90	0.85
	5	0.69	1.46	1.34
	5	0.61	1.05	1.24

If the method is specific the S/I ratio must only arise from comp. F. and the method offers a good criterion of purite as inhomogenity of the spot is very easy to detect and it is very unlikely indeed that the ester of any other substance will move exactly as the ester of comp. F when this has an Rf of 0.3—0.5. In Table IV are shown some data from double analyses on dog and human plasma. No extra steroid was added to the plasma. The spots in the final chromatograms were divided in an upper and a lower half and the S/I ratio determined in each of them. The percentage difference between the two determinations of the S/I ratios of the spots are all except two within the s. d. determination. These two are both within twice the s. d. of the counting procedure of these R-values (taken from Fig. 1).

Table V. *Determinations of Comp. (17- hydroxycorticosterone) in plasma of d and humans (students) and the recovery of steroid added to plasma before the extracti*

	Plasma ml	µg Comp. F found preformed	µg Comp. F added	Per- centa recov
dogs	10	0.23	1.15	89
		0.22		
	6	0.13		
	10	0.28	0.66	84
		0.30		
	10	0.33	0.66	86
	10	0.24	0.575	90
		0.245		
	10	0.25	0.575	89
humans	10	1.19	0.58	79
		1.22		
	10	1.26	0.58	87
	5	0.845	0.58	82
		0.86		
	5	0.876	0.58	90
	5	0.51	0.58	77
		0.50		
	5	0.49	0.58	81
	5	0.53	0.58	85
		0.51		
	5	0.49	0.58	95
	5	0.69	0.55	70
		0.65		
	5	0.61	0.55	100
	5	0.28	0.45	83
		0.295		
	5	0.31	0.45	104

In Table V are given the results of covery of comp. F added to plasma fr dogs and humans (students). The d were mongrels and the blood sample w taken 5—10 minutes after their remo from the cage. The blood was taken fr the students about 10 a.m. The blood w

rizined and centrifuged immediately it was taken. Two analyses of each na were run for determination of pre- ed Comp. F and two with the steroid d before extraction with chloroform.

e average recovery of Comp. F added lasma is 86 per cent in these experi- s, shown in Table V, and the scatter is t what is to be expected with the stan- deviation calculated for all double minations made so far with this proce- As the recovery of comp. F added to na extract was about 95—100 per cent, oss of steroid must occur in the extrac- procedure and not in the process of fication in spite of the great many ances present there.

far no other extraction procedure has tried and it can only be stated that the sis is specific, accurate and with good ery, but to what extent all the pre- ed Comp. F is determined is impossible without the use of ^{14}C labelled steroid. ever Simpson & Tait (1955) obtained 97 per cent recovery of added ^{14}C-label- ydrocortisone with a chloroform extrac- similar to the one used here. It there- seems reasonable to assume that the ery of preformed comp. F is equal to ecovery of that added.

The analysis in general

e great advantage of the reagent used e simplicity of the counting procedure he simplicity of the calibration. The od of Avidi *et al.* (1954), employing me principles but acetic acid anhydride e reagent, did not lead to satisfactory s because it seems difficult to get rid e excess reagent, and the counting procedure is difficult, involving determinations of ^{14}C and ^{3}H in two different counters. The geometry therefore will play a significant rôle in limiting the accuracy of the method. This is not the case when S/I ratios are the only activity determinations entering the calculations, because the geometry will influence the $\bar{s}$ counts in just the same way as the $\bar{c}$ counts.

The full range of the method is not yet established, but it seems likely that other corticosteroids present in plasma can be determined just as well as comp. F and that the sensitivity of the method can be increased about tenfold by using a counter more sensitive to ^{35}S and a higher specific activity of the "pipsan ^{35}S".

SUMMARY

A method using the principle of isotopic derivation and p-iodophenylsulfonylic acid anhydride as the reagent labelled with ^{35}S or ^{131}I has been devised for the analysis of 17-hydroxycorticosterone in plasma of dogs and humans. The range of the method is 0.2—1.5 μg in which interval the s. d. has been found to be ± 6 per cent; more steroid can easily be determined with the same or higher accuracy. The loss in the extraction procedure is about 14 per cent. When the substance is added to plasma extract it is recovered as the ester almost quantitatively. The method permits an effective control of its specificity in each individual sample.

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SPECTROPHOTOMETRIC DETERMINATION OF HEMOGLOBIN OXYGEN SATURATION IN HEMOLYZED WHOLE BLOOD

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Spectrophotometric methods for determining the oxygen saturation of hemoglobin are being used to an increasing extent. Since the fundamental publication of Drabkin & Schmidt (1945), a number of methods have appeared (Nahas, 1951; Hickam, Pryor, Page & Atwell, 1951; Roos & Rich, 1952; Wade, Bishop, Cumming & Donald, 1953; Klungsøyr & Støa, 1954; Stainsby, Fales & Lilienthal, 1955).[1]

In comparison with the manometric method of Van Slyke & Neill (1924) the spectrophotometric methods have the advantages of being simpler and much more rapid and requiring smaller blood samples.

The normal values for arterial oxygen saturation are usually found to be somewhat higher (98.0—98.6 per cent) with the spectrophotometric methods (Drabkin & Schmidt, 1945; Hickam *et al.*, 1951; Lambertsen, Bunce, Drabkin & Schmidt, 1952; Klungsøyr & Støa, 1954) than with Van Slyke & Neill's manometric method (97.0—97.9 per cent) (Comroe & Walker, 1948; Wood, 1949; Wood & Geraci, 1949; Lambertsen *et al.*, 1952; Dempsey & Wilson, 1954).

In choosing a spectrophotometric method it seemed to us that the methods of both Nahas (1951) and Klungsøyr & Støa (1954) would fulfill our requirements of accuracy and rapid, simple performance and would be well suited to our spectrophotometer (Beckman Model DU). We therefore found it desirable to make a comparison between a slightly modified version of Nahas' very simple and inexpensive plastic cuvette and a considerably more expensive and complicated glass cuvette, Klungsøyr & Støa's modification (1954) of Drabkin & Austin's cuvette (1935).

Since both Nahas and Klungsøyr & Støa found rather marked disagreement in some of their analyses between the spectrophotometric and manometric results, we decided to make a comparison with the manometric method at the same time.

In the present investigation the optical density was measured at 576 and 560 mμ, a maximum and a minimum in the absorption curve of HbO_2. Calculation of the oxygen saturation was made as follows (Drabkin, 1950):

$$X = \frac{\frac{DHbO_2576}{DHbO_2560} \div \frac{D\,576}{D\,560}}{\frac{D\,576}{D\,560}\left(\frac{DHb560}{DHbO_2560} \div 1\right) + \frac{DHbO_2576}{DHbO_2560} \div \frac{DHb576}{DHbO_2560}}$$

X is that part of the total pigment present as reduced Hb; $DHbO_2$ and DHb are the optical densites for fully oxidized and reduced hemoglobin respectively at the wave lengths employed. When the constants $DHbO_2576/DHbO_2560$, $DHb560/DHbO_2560$ and $DHb576/DHbO_2560$ are determined beforehand and D576 and D560 are measured in the blood sample tested, X can be calculated and the oxygen saturation will be 100 (1—X).

In order to obtain accurate spectrophotometric results it is especially important that the constant $DHbO_2576/DHbO_2560$ and the ratio D576/D560 in the blood to be analysed

[1] After this paper had gone to press: Tsao, Sethna, Sloan & Wyngarden (1955) and Holling, Mac Donald, O'Halloran & Venner (1955).

are determined as accurately as possible. Both are influenced by COHb and by a pronounced increase in serum lipids, but not by an increase in serum color, even if large. Both are temperature dependent (Refsum, 1956).

The influence of varying concentration of "inactive" Hb is questionable. Van Slyke, Hiller, Weisiger & Cruz (1946) report an average of 1.3 per cent "inactive" Hb in the total pigment in freshly drawn venous blood, but Comroe & Walker (1948) and Dempsey & Wilson (1954) doubt the presence of significant quantities in arterial blood.

METHODS

In these investigations we used heparin blood from young, healthy non-smokers who had ingested a light, low fat content breakfast 2—3 hours before the samples were taken. To avoid the disturbing influence of fibrin precipitation during spectrophotometry, it was important to draw the blood rapidly and to mix it thoroughly with heparin. To reduce the influence of possible "inactive" Hb to a minimum and to avoid a differential effect on the two analytical methods by a possible reversion of "inactive" Hb to active Hb, both the spectrophotometric and manometric investigations were performed at the same time, at least two hours after the samples were drawn.

Blood for the comparative investigations was drawn without stasis in a Luer syringe from the cubital vein with the usual anaerobic technique. Varying oxygen saturation was obtained by prewarming the arm in a water bath before the blood was drawn. Aliquots were taken from the original blood sample immediately before the analyses were begun.

Spectrophotometric method

Cuvettes: Klungsøyr & Støa's glass cuvette, depth 0.07 mm (Klungsøyr & Støa, 1954). For injection into the cuvette we used a straight, cut off Record cannula, connected with the injection tube of the cuvette by a bored rubber stopper.

Nahas' lucite plastic cuvette, depth 0.1 mm (Nahas, 1951). Instead of the angular canals through the plastic for filling and emptying, slots were ground directly into the analysis chamber in the plastic along the metal gasket.

Technique: Except for the details mentioned below, the analyses were performed as described by Klungsøyr & Støa (1954). After a 10-minute *equilibration* of the blood sample *in a 20° C water bath,* filling of the Luer syringe with 0.1 ml of 30 per cent saponin (Merck) per 5 ml blood, thorough *mixing for 1 minute,* and injection into the cuvettes, the optical density was *immediately* read at 576 and 560 mμ in a Beckmann spectrophotometer Model DU, aperture 0.04 mm.

Determination of the spectrophotometric constants: 5—7 ml non-hemolyzed blood was rotated 15 min. in a 300 ml tonometer, periodically flushed with fresh air. After the drawing of the blood into the Luer syringe and further analytical procedure as described above, $DHbO_2576$ and $DHbO_2560$ were read. Then the rest of the hemolyzed blood was reduced by thorough mixing with 6 mg $Na_2S_2O_4$ per ml blood and DHb576 and DHb560 were read in the *same* cells.

Comments on calculation: In all determinations, both with the glass and the plastic cuvette, the optical density was read in two cells. All readings were carefully corrected according to the blank readings at 576 mμ of the cells employed, filled with water. The spectrophotometric ratios were taken as the average of the two ratios calculated for each cell separately.

Manometric method

The manometric analyses were carried out according to Van Slyke & Neill (1924) but without special absorption of carbon dioxide, using the following reagent: 16 g $K_3Fe(CN)_6$, 30 g $Na_2B_4O_7 \cdot 10\,H_2O$ and 5 g saponin per liter solution (Van Slyke, 1943). Corrections for dissolved oxygen were made according to Sendroy, Dillon & Van Slyke (1934) and Comroe (1950).

RESULTS AND DISCUSSION

Determination of spectrophotometric constants

The results are presented in Table I. They show that under the described analytical procedure these constants can be determined with great accuracy with both types of cuvette. In some determinations corrections were made for small deviations of the temperature from 20° C. The somewhat larger variations in the ratios $DHb560/DHbO_2560$ and $DHb576/DHbO_2560$ can be ascribed to accidental small differences in the concentration of total pigment and/or small variations in the depth of the cells from the first reading to the second.

Table I. *The constants for calculation of hemoglobin oxygen saturation in hemolyzed whole blood determined by measuring the optical density at 576 and 560 mμ in fully oxygenated and reduced blood in the glass and plastic cuvette at 20° C.*

Spectrophotometric ratio	Type of cuvette	Number of determinations	Mean	S.D.
$\frac{DHbO_2576}{DHbO_2560}$	Glass	18	1,806	0.002
$\frac{DHbO_2560}{DHb576}$	Plastic	10	1.807	0.004
$\frac{DHb576}{DHbO_2560}$	Glass	10	1.094	0.005
$\frac{DHbO_2560}{DHb560}$	Plastic	10	1.094	0.005
$\frac{DHb560}{DHbO_2560}$	Glass	10	1.470	0.005
	Plastic	10	1.471	0.005

When the constants are substituted in the formula, the oxygen saturation determined with the glass cuvette is:

$$100 \cdot \left[\frac{1.47 \cdot D\ 576 / D\ 560 \div 1.094}{0.47 \cdot D\ 576 / D\ 560 + 0.712}\right] \text{ per cent}$$

Comparison of spectrophotometric and manometric results

The results are presented in Table II. They show that at both high and low degrees of oxygen saturation there is good agreement among the results of the methods used. The mean difference of the spectrophotometric values from the manometric is — 0.2 per cent for the glass cuvette and + 0.6 for the plastic cuvette, with a standard deviation of 1.1 per cent for both cuvettes.

The differences between the spectrophotometric and the manometric values in single analyses may be due in part to small accidental variations in the composition of the blood samples (COHb, "inactive" Hb, serum lipids) which have a differential effect in the two types of analyses. It should be stressed that faulty adjustment of the temperature of the blood samples yield inaccurate spectrophotometric values.

The results obtained both with the glass and the plastic cuvettes are satisfactory. The

Table II. *Hemoglobin oxygen saturation in per cent determined by spectrophotometric and manometric method in 19 samples.*

Manometric method	Spectrophotometric method	
	Glass cuvette	Plastic cuvette
20.3	20.1	21.0
34.7	34.1	34.8
47.5	47.3	47.9
54.0	52.2	53.6
57.9	58.2	58.5
72.4	71.8	72.2
82.6	84.3	84.7
83.6	83.0	84.9
84.4	83.7	84.3
87.2	87.4	88.2
87.5	85.2	85.9
90.8	92.9	93.3
92.2	91.1	92.1
92.6	92.4	93.6
93.0	94.6	94.9
93.1	94.0	95.0
93.7	93.8	94.3
95.0	94.0	95.0
96.9	95.9	96.2
Mean 76.8	76.6	77.4

consistent difference between the values obtained with the two types of cuvette can be explained as follows. Following the hemolysis with saponin, there is often a gradual reduction of the oxygen saturation. This process continues unaltered inside the glass cuvette, as shown by the *decrease* in the ratio D576/D560. Inside our plastic cuvette however, the optical density of the blood undergoes alteration, which corresponds to oxidation, as shown by the continuous, relatively rapid *increase* in this ratio. Consequently the time which elapses both between hemolysis and reading and injection into the cuvette and reading is significant. Except for the fact that injection and reading were performed as quickly as possible after hemolysis, no attempt was made to correct for these phenomena in the present investigation.

The mean difference between the duplicate readings is 0.18 per cent for the glass

cuvette and 0.32 per cent for the plastic, with a standard deviation of 0.15 and 0.30 per cent respectively. The larger deviations with the plastic cuvette may be due to the depth of this cuvette (0.1 mm) which gives unfavorably high values of optical density at the wave lengths employed and thus greater inaccuracies in the readings; they may also be due to small defects in the plastic and to the fact that this cuvette is more difficult to keep clean than the glass cuvette, as often observed in the blank readings. In evaluating the two cuvette types employed it should also be mentioned that that the depth of the plastic cuvette is dependent on temperature.

SUMMARY

Hemoglobin oxygen saturation has been determined both manometrically, using the method of Van Slyke & Neill, and spectrophotometrically, using two types of cuvette: one, a modification of Nahas' plastic cuvette, and the other, the Klungsøyr & Støa modification of Drabkin & Austin's glass cuvette. Under the described analytical procedure good agreement has been found amoung the results of 19 different blood samples having various values of hemoglobin oxygen saturation. With the plastic cuvette somewhat higher values have been found than with the glass cuvette. Sources of error in the spectrophotometric methods have been discussed.

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PRODUCTION OF SERUM FROM SMALL AMOUNTS OF BLOOD

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In a study on the serum proteins in mice it proved fairly easy to procure small amounts of blood without significant after-bleeding. On the other hand, the procedure used initially to obtain serum was not reliable, the clot quite often adhering so firmly to the wall of a capillary tube closed at one end that centrifugation gave incomplete separation.

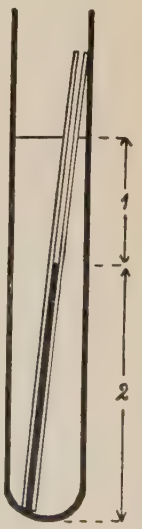
Therefore, the following method was worked out: Cut one or two millimetres off the tail with scissors and draw the blood immediately into a capillary tube. Its lumen should preferably be one square mm, as this gives a length of the blood column in millimetres equal to its volume in cubic mm. Hold the capillary tube in a vertical position and draw the first small drop of blood into its lower opening. Later, as it becomes more difficult for the capillary force to draw the blood, change gradually to an oblique or horizontal position.

When a sufficient amount of blood has been obtained, leave the capillary tube in the horizontal position for 1/2 to 2 hours at room temperature or in the refrigerator. Then place it — blood column down — in an ordinary centrifuge tube containing a mixture of 10 parts — by volume — of liquid paraffin and 3 parts of carbon tetrachloride. The density of this mixture should be between 1.045 and 1.055. During the subsequent centrifugation — 10 minutes in an ordinary laboratory centrifuge — the mixture displaces the serum, which has a lower density, from the clot. As the latter is of a density higher than the mixture it remains adherent to the capillary wall or is centrifuged down. After centrifugation, the interface between the serum and the mixture is seen completely free of the clot.

Fish the capillary out, wipe it off with filter paper, and cut it in two immediately above the interface. The serum may then be directly sucked out and measured with a constriction pipette (of the Carlsberg type).

SOME COMMENTS ON THE EXPERIMENTAL CONDITIONS

1. The amount of serum obtained does not seem to depend on whether clotting and contraction of the clot take place at room temperature or at + 5°.



2. The clotting and contraction time must be a minimum of half an hour. If up to 18 hours elapse before centrifugation, there is a risk of a reduced yield and slight hemolysis, especially in that part of the serum column which borders on the interface.

3. Centrifugation must be performed immediately after the capillary tube has been placed in the paraffin-carbon tetrachloride mixture. Otherwise, hemolysis may occur in

[1] The expenses of this investigation were defrayed by a grant from The Danish State Research Foundation.

the border layer. On the other hand, the capillary tube may without risk be left in the mixture after centrifugation.

4. The density of carbon tetrachloride is about 1.60, and only 0.8 g may be dissolved in 1000 g of water at 20°. The liquid paraffin, which is entirely insoluble in water, had a density of about 0.89. In fixing the density of the mixture due regards was paid to the fact that a serum — with a protein content of 13 g per 100 ml — has a density of 1.045 whereas the density of the clot is always bound to be considerably higher than 1.055 (the density of red blood cells is 1.100 ± 0.006 (E. Ponder, J. Biol. Chem. 144, 333—338, 1942)).

5. The centrifuge tube is filled with the mixture at least to the level shown in the figure, if the interface is to be definitely free of the clot. The mixture can be used an unlimited number of times, if only the correct density has been secured. Impurities may be filtered off.

In most cases the method was used in dealing with blood samples of 20 to 50 mm^3 in capillary tubes with lumina of about 1 mm^2. If normal human blood is used then the yield of serum is 45—55 per cent. However, the method has also been used for samples as small as 2 mm^3 in capillaries with lumina of only 0.045 mm^2 — with a yield of about 45 per cent.

Judged by paper electrophoresis experiments the procedure does not entail any alteration in the serum proteins.

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DETERMINATION OF URINARY UROPORPHYRIN BY A DIRECT EXTRACTION METHOD

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Coproporphyrin present in urine or similar fluids may readily be determined by direct extraction procedures. Ether containing acetic acid is the organic solvent usually used for extraction, after which the porphyrin is returned to aqueous acid and estimated either by fluorimetry or spectrophotometry (Saillet, 1896; Fikentscher, 1932; Brugsch & Kubowitz, 1953). More recently Schwartz, Zieve & Watson (1951) have described a procedure for determining coproporphyrin in urine in which the extractant is ethyl acetate.

The determination of uroporphyrin, on the other hand, has always presented considerable difficulty since it is not extractable by ether, and adsorption techniques have, of necessity, been resorted to, but they have not been entirely satisfactory from the point of view of ease of manipulation and quantitative recovery (Rimington, 1943; Sveinsson, Rimington & Barnes, 1949; Brugsch, 1949; Askevold, 1951; Lockwood, 1953; With & Petersen, 1954). The ether-insoluble porphyrin present in acute porphyria urines ("Waldenström porphyrin"), which is probably a mixture of uroporphyrin isomers together with other porphyrins such as a hepta-carboxylic porphyrin, was found by Waldenström, Fink and Hoerburger (1935) to be extractable into ethyl acetate within the narrow pH range 3.0—3.2.

Recently Dresel & Tooth (1954) specified conditions under which uroporphyrin as well as Waldenström porphyrin will pass quantitatively into ethyl acetate from solutions containing low concentrations of these porphyrins. This was contrary to the findings of Waldenström, Fink & Hoerburger (1935), who were unable to extract uroporphyrin from aqueous solutions by ethyl acetate. It seemed possible that quantitative extractions are obtained only with low concentrations of uroporphyrin I, and that a difference between the behaviour of the two isomers would be observed at higher concentrations such as those present in the urines of certain pathological conditions. Further, it has been suggested (Watson, Berg & Hawkinson, 1955) that recoveries from urines might differ from those obtained with pure solutions. We have therefore performed several experiments with high concentrations of uroporphyrins I and III (Waldenström porphyrin which, when tested by the paper chromatographic method of Falk & Benson (1953), behaved as uroporphyrin III only), added to aqueous solutions and to normal urines. In no case have we been able to observe any significant difference between the behaviour of uroporphyrin I on the one hand and Waldenström porhpyrin on the other, when both porphyrins were tested under identical conditions. Thus, for example, at a concentration of 10 mg/l in normal urine, quantitative extraction of both porphyrins with ethyl acetate was possible after the addition, to 10 ml of urine, of 2 ml of 10 per cent (2.8 *N*) HCl and 6 ml of saturated Na acetate and subsequent adjustment to pH 3.2. How-

ever, similar treatment of normal urine containing 30 mg/l of uroporphyrin resulted in partial precipitation of both types of porphyrin, and obviously, therefore, extraction into ethyl acetate was incomplete. A concentration of 10 mg/l is of the order which might be expected in a typical acute porphyria urine, containing the Waldenström type porphyrin. On the other hand, urines from cases of congenital porphyria may contain much higher concentrations. We recently found a concentration of some 150 mg/l of uroporphyrin I in a typical case. This porphyrin was precipitated almost completely on adjusting the urine to pH 3.2; when, however, the urine was diluted 15 times with normal urine, the recovery of uroporphyrin, using ethyl acetate as the extracting agent, was as high as when cyclohexanone (see below) was employed. We feel, therefore, that the differences in solubility observed in various laboratories were due to the fact that the isomers were handled at different concentrations. We do, however, confirm the observation of Waldenström *et al.* (1935) that crystalline uroporphyrin I will not dissolve in glacial acetic acid, whereas Waldenström porphyrin or uroporphyrin III (from turacin) are readily brought into solution by this acid. Finally, it is of interest that the presence of acetic acid is not essential for passage of uroporphyrin into ethyl acetate. When a solution of uroporphyrin I in weak alkali was carefully adjusted to pH 3.2 with dilute HCl, the majority of the porphyrin, as judged by fluorescence, passed into the ethyl acetate layer in one extraction.

Kennedy (1954) observed that many porphyrins, including uroporphyrins, will pass readily from suitably acidified solutions into cyclohexanone and suggested that this might form the basis of a quantitative procedure. We have made a study of the phenomenon as a result of which we present a technique for the determination of uroporphyrin I or Waldenström porphyrin present in urine. It is reasonably rapid and affords recoveries of added uroporphyrin (either isomer) ranging from 100 per cent at the level of 1 mg/l to 80 per cent at the 100 μg/l level. At still lower concentrations, recoveries are not quite so good, 66 per cent to 68 per cent being recovered from 20 μg/l of urine. The 'blank' determinations carried out upon the normal urine without any addition confirm the statements of Nicholas & Rimington (1949), Schwartz (1953), Lockwood (1953), and With & Petersen (1954) that normal urine does contain small amounts of uroporphyrin. We find the concentration to be of the order of 5—20 μg/l. For the determination of such quantities, a small modification is recommended of our general procedure for pathological urines (see below). Should very large quantities of uroporphyrin be present in urines ($>$ 1 mg/l) the concentration may be determined without great error by diluting with HCl to obtain a final concentration of 0.5 N, reading the density at the Soret maximum and also at 430 mμ and 380 mμ and then applying the correction formula of Rimington & Sveinsson (1950).

MATERIALS

Uroporphyrin I. (Octa-methyl ester M.P. 293°) was isolated from congenital porphyria urine (Rimington & Miles, 1951).

"Waldenström porphyrin". (Methyl ester M.P. 260°) was pooled material isolated from urines of cases of acute porphyria (Nicholas & Rimington, 1953). When tested by the paper chromatographic method of Falk & Benson (1953) it behaved as uroporphyrin III only.

Cyclohexanone. The commercial product was distilled under reduced pressure and the fraction with constant B. P. was used without further purification.

Light petroleum. B. P. 40—60°. Commercial product.

For recovery experiments the porphyrin esters were hydrolysed by leaving in contact with 25 per cent HCl (ca 7 *N*) for 36 hours at room temperature and then evaporated to dryness in vacuo over NaOH and P_2O_5. The free porphyrins were dissolved in a small volume of 0.1 *N* $NaHCO_3$ and the exact concentration determined spectrophotometrically after suitably diluting a sample with 2 per cent HCl (ca 0.55 *N*).

METHOD AND RESULTS

The standard method finally adopted for pathological urines, and which gave the results shown in Table I, was as follows.

50 ml of urine were shaken twice with 10 ml of glacial acetic acid and 100 ml of ether to extract the coproporphyrin. The combined ether layers were shaken once with 30 ml of saturated sodium acetate to remove any uroporphyrin which might have been carried over and this washing was added to the aqueous layer. It was found that less than 0.2 per cent of the coproporphyrin present in the ether is removed under these conditions. Should the uroporphyrin concentration in the urine be very high, several aqueous washes of the ether may be necessary; the usual precautions of pH control should then be taken to avoid return of the coproporphyrin into the aqueous layer. For extraction of the uroporphyrin, the aqueous layer was next adjusted to pH 1.5 with conc HCl, using a pH meter, and then extracted twice with 50 ml of cyclohexanone.[1] An equal volume (100 ml) of light petroleum (B.P. 40—60°) was added to the combined cyclohexanone extracts, since uroporphyrin passes more readily into dilute HCl from this mixed solvent than from cyclohexanone alone. Complete removal of the uroporphyrin was then achieved by repeated extractions with small amounts of 2 per cent HCl until the aqueous extract was non-fluorescent. It should be mentioned that a small aqueous layer separated out on addition of light petroleum to the cyclohexanone; this was added to the acid extracts since it may contain some uroporphyrin. For the determination of uroporphyrin in the acid extract, readings were taken in a Unicam spectrophotometer, model SP 500, at the Soret maximum lying at 404—405 mμ, and also at 380 mμ and 430 mμ, and the correction formula described by Rimington & Sveinsson (1950) applied. $E^{1\,0/0}_{1\,cm}$ was taken as 6517.

Some results obtained by the use of this method are illustrated in Table I.

A determination of the apparent uroporphyrin content of the urine to which no addition had been made was carried out alongside every recovery experiment. The recoveries of added porphyrin are expressed in two different ways. The first column of

Table I. *Recovery of uroporphyrin I added to normal urine.*

Porphyrin added (μg.)	«Blank» *i.e.* apparent uroporphyrin content of sample (μg.)	Recovery	
		Corrected for blank (in per cent)	Not corrected for blank (in per cent)
I. Concentration level 1 mg/l. (50 ml urine. used).			
49.6	0.27	98	99
51.5	0.25	94	95
51.5	0.25	99	100
52.1	—0.30	92	91
52.1	—0.30	90	89
49.9	0	93	93
49.9	0	94	94
II. Concentration level 100 μg/l. (50 ml of urine used).			
5.0	0.24	89	95
4.8	0.14	73	76
4.8	0.14	76	77
5.1	—0.48	81	72
5.1	—0.48	81	72
5.0	—0.10	87	87
5.0	—0.10	89	87
III. Concentration level 20 μg/l. (250 ml of urine used).			
5.0	—0.37	66	58
5.0	—0.37	68	61
5.1	—1.67	67	33
5.1	—1.67	68	35

[1] Two such extractions were sufficient for concentrations of uroporphyrin such as those shown in Table I. In cases where the porphyrin content is very much higher, three to four extractions with cyclohexanone may be necessary for complete removal of the uroporphyrin from the urine.

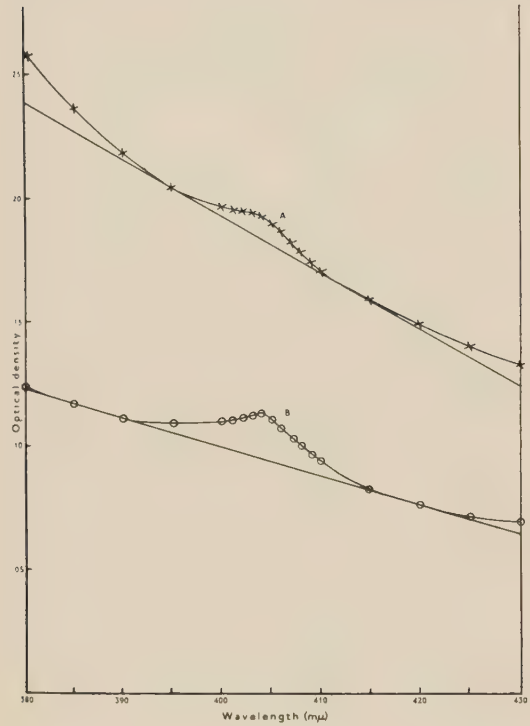

Fig. 1. Optical densities, over the range 380—430 mμ, of final acid extracts from the same urine obtained by (A) the standard procedure and (B) the modified procedure for small quantities of uroporphyrin in which ether instead of light petroleum is added to the cyclohexanone extract. The straight lines represent the ideal condition demanded for application of the correction formula of Rimington & Sveinsson (1950). Note the better approximation in B and also the lower total absorption.

Table I gives the true recoveries, after allowing for the 'blank' reading of the urine alone; in the second column no such allowance has been made.

It will be seen that at the highest uroporphyrin concentration of mg/l the recoveries are good and there is no significant difference between those calculated respectively by including and omitting the 'blank' values. At the 100 μg/l level, inclusion of the 'blanks' gives recoveries of 73—89 per cent and omitting them a range of 72—95 per cent. At the lowest level tested, 20 μg/l, considerable differences are introduced by including on omitting the 'blanks'. Thus true recoveries (blanks allowed for) range from 66 per cent to 68 per cent, but with blanks omitted the range is 33—61 per cent. It will be seen from inspection of Table I that the blanks are sometimes negative, which can only mean that the conditions required by the correction formula do not obtain, namely a *uniformly* rising or falling absorption between 430 mμ and 380 mμ due to impurities in the acid extract (see Rimington & Sveinsson, 1950). In order to study the point more closely we recorded the spectral absorption curve of some 'blank' acid extracts over this range (Fig. 1); a distinct curvature is evident which would lead to error when the amount of uroporphyrin undergoing measurement is small.

Modifications of the procedure were sought which would remedy this situation and it was found that by adding ether instead of light petroleum to the cyclohexanone extracts the total absorption due to impurity passing into the acid phase was greatly reduced. It was necessary however to use 5 per cent HCl (instead of 2 per cent) to extract the porphyrin from the organic phase. The example given in Fig. 1 will show that the linearity of the absorption of such a blank was also much improved. For determining very small quantities of uroporphyrin, such as are found in normal urine, we therefore recommend this modification and some results obtained with its use are given in Table II. It is rather more time-consuming since the separation of the aqueous acid phase is not so rapid as in the standard procedure.

Since urines from cases of acute porphyria may contain both uroporphyrin and porphobilinogen, the possibility of conversion of porphobilinogen to uroporphyrin during the extraction procedure was also investigated. 1 mg porphobilinogen was added to 10 ml of urine, and the standard

rocedure followed. The final acid extract, hich was faintly fluorescent, contained .63 μg uroporphyrin. There was thus a onversion of less than 0.1 per cent of the dded porphobilinogen to uroporphyrin. Since, however, the porphobilinogen concentration in acute porphyria urine may mount to 100 mg/l, the possibility exists hat some 100 μg/l uroporphyrin could arise luring the determination, if this method s used.

The method presented here is probably f greatest value in cases of mild or susected porphyria or porphyrinuria, where rapid, but sensitive, and reasonably acurate method for the determination of roporphyrin is requiered. It has the great dvantage that uroporphyrin is determined irectly and not by difference. One specimen of urine suffices for determination of oth coproporphyrin and uroporphyrin.

As an example of the order of agreement ound by application of (1) the present yclohexanone procedure (2) the ethyl cetate extraction method and (3) direct pectrophotometry to urines containing nuch uroporphyrin I or III), some typical esults are assembled in Table III.

Table II. *Uroporphyrin content of normal urines determined by modification of standard procedure (see text).*

50 ml urine analysed; correction formula of Rimington & Sveinsson applied to readings on the 5 per cent HCl extract.

Urine Specimen	Uroporphyrin (μg/l.)
1	2.7
2	3.5
3	7.4
4	4.6
5	5.0
6	4.0
7	14.6

Table III. *Comparison of analytical methods applied to pathological urines containing high concentrations of porphyrin.*

Urine	Uroporphyrin (mg/l) by		
	Cyclohexanone method	Ethyl acetate method	Direct spectrophotometric method*)
Mr. W: Congenital porphyria	123.8	121.2	167.5
Mrs. A: Acute porphyria	10.56	10.56	11.18
Mrs. Q: Acute porphyria	3.02 2.90 2.87**)		

*) Includes coproporphyrin.
**) Shaking of cyclohexanone extract with 0.005 per cent Iodine included.

SUMMARY

1. A method is described for the determination of uroporphyrin (both I and III isomers) in urine or similar fluids. It involves extraction by cyclohexanone at pH 1.5 after prior removal of coproporphyrin by ether-acetic acid.

Recoveries were 66—68 per cent, at 20 μg/l, 73—89 per cent at 100 μg/l, and 90—100 per cent at 1 mg/l.

2. Experiments are reported on the extractability of both uroporphyrin I and Waldenström porphyrin from urine by ethyl acetate at pH 3.0—3.2.

ACKNOWLEDGMENT

We wish to thank Dr. G. Y. Kennedy for much helpful information concerning his use of cyclohexanone for the qualitative extraction of porphyrins.

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Scandinav. J. Clin. & Lab. Investigation 79—80, 8, 1956.

A NEW METHOD FOR THE EXTRACTION OF UROPORPHYRIN I FROM MOLLUSC SHELLS

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(Received for publication December 12, 1955)

The usual method for the extraction of uroporphyrin I from mollusc shells is that of Fischer and Haarer (1932), which involves the preparation of large volumes of methanol saturated with dry hydrogen chloride at 0° C. This is a formidable task in most laboratories, and an alternative method was sought which would be quicker and less expensive. After solution of the powdered shells in dilute hydrochloric acid, a solvent was required which would extract the uroporphyrin without interference from the calcium chloride formed in the solution process. After some considerable experiment, it was found that cyclohexanone was an admirable solvent for this purpose, extracting uroporphyrin, protoporphyrin and coproporphyrin from their solutions in dilute hydrochloric acid with facility.

MATERIALS

Shells

The starting material was Persian Lingah shell, *Pinctada vulgaris*, kindly supplied by Mr. F. Singleton, Pearl Cutter, Sheffield, and ground to a fine powder by the courtesy of Messrs Batchelors Ltd. Sheffield.

Cyclohexanone

This was the pure commercial product as supplied by May and Baker Ltd. of Dagenham. It was colorless, and had a boiling range of not less than 90 per cent between 153—157° C. All other reagents were of Analar quality.

METHOD

10.79 kg of the powdered shells was added in small amounts to 10 litres of 10 per cent w/v hydrochloric acid, shaking gently, and adding a few drops of sec-octyl alcohol (capryl alcohol) to reduce frothing. The whole operation was carried out in a 20 litre flask, and as the acid became spent, the solution was decanted and fresh acid added. When all the shell had been added, the mixture was allowed to stand overnight in the ice-chest, and then filtered at the pump from insoluble residue. The filtrate was brought to about pH 2.0 by the addition of concentrated hydrochloric acid, and extracted with cyclohexanone, portions of 3 liters at a time being shaken with 500 ml of the solvent in a 5 liter separating funnel.

When the cyclohexanone epiphases were no longer red-fluorescent in ultra-violet light (Osira 125 watt black-glass lamp), they were pooled and washed repeatedly with 2 per cent sodium chloride (6 washes of one-third of the volume each) and water (12 washes of one-third of the volume each). This thorough washing was essential to remove the calcium salts. The extract was then dried roughly by filtration through thick cyclohexanone-soaked papers (Green's No 904½), and the solvent evaporated under reduced pressure, keeping the temperature as low as possible.

When the volume of the porphyrin solution had reached approximately 50 ml, the residue was filtered through a fast cyclohexanone-soaked paper into 2.5 liters of ether, and the mixture left in the ice-chest (under a greased bell-jar and ground-glass plate) to flocculate overnight. The supernatant ether was carefully decanted, and the precipitate washed in the centrifuge with ether several times. The crude uroporphyrin was then esterified by dissolving it in 100 ml of methanol saturated at 0° C. with hydrogen chloride, and left at room-temperature for 48 hours. The ester was recovered by extraction with chloroform in the usual way, purified by chromatography on columns of alu-

mina and magnesia Grade III (Nicholas 1951), and crystallised from chloroform and examined by the Chu, Green and Chu (1951) and the Falk and Benson (1953) techniques. This revealed the presence of a trace of uroporphyrin III.

The yield

of red-brown needles from 10.79 kg of starting material was 0.274 g, melting at 289.5° C.

This method saves the time which would be spent in saturating large volumes of methanol with dry hydrogen chloride — several weeks — and also saves the expense of this methanol and much chloroform.

SUMMARY

A new method is described for the extraction of uroporphyrin I from acid solution by cyclohexanone. This method saves time and cost of materials.

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Scandinav. J. Clin. & Lab. Investigation 81—82, 8, 1956.

PLASMA FREE FATTY ACIDS IN DIABETIC ACIDOSIS AND STARVATION

(Received for publication January 27, 1956)

Reports of increased electrophoretic mobility of serum lipoproteins in diabetic acidosis in the dog (Fasoli, 1954) and human beings (Dangerfield, 1955) prompted the writer to determine the free fatty acids in plasma in this condition, it being known that free fatty acids can influence the mobility of lipoproteins (Laurell, 1955; Gordon, 1955). In view of the similarities in the lipid metabolism in diabetes and starvation, the investigation was extended to include starving, apparently healthy human volunteers.

METHOD

The free fatty acids were determined according to Grossman (1955), 2 ml of EDTA-2Na plasma being used for every determination. The analysis was started within 20 minutes of the collection of the sample. Centrifugation was usually carried out at 0° C. Analysis of the method showed that the procedure determines 90—100 per cent of aliphatic fatty acids with 14—18 carbon atoms, 10—80 per cent of those with 8—12 carbon atoms and none of those with less than 6 carbon atoms nor β-oxibuturic acid. S. D., as calculated on the basis of the results of 16 analyses of one and the same serum sample, was found to be δ=0.019 mEq/litre. Normal value, as found for 15 medical students: 0.63 mEq/litre S. D. δ=0.12 mEq/litre.

RESULTS

The results obtained in a case of diabetic acidosis are given in Table I. In 2 other cases the initial concentration of the fatty acids was found to be 2.62 and 3.02 mEq/litre, which dropped to 0.70 and 0.64 mEq/litre during insulin therapy. The values found are not sufficient to produce any appreciable increase in the electrophoretic mobility of the lipoproteins. The results obtained with serum collected from a healthy subject during starvation are given in Table II. Three other experiments showed a maxium increase of free fatty acids from 0.59 to 2.03, from 0.61 to 1.33, and from 1.11 to 2.76 mEq/litre.

Determination of free fatty acids after incubation for one hour at 37° C. was performed at one of the cases of diabetic acidosis and repeatedly during the starvation experiments. No increase in free fatty acids was noted as compared with immediately analysed samples treated in the cold. It

Table I. *Case of diabetic acidosis. Time= interval since beginning of insulin theraphy.*

Time hours	Plasma free fatty acids mEq/litre	Plasma HCO_3 mEq/litre	Blood sugar g/100 ml
0		7.2	0.40
0.25	2.44		
2.5	1.92	6.8	0.26
6.5	0.56	18.2	0.36
8.5		24.3	
17.5	0.47		0.24

Table II. *Starvation experiment. Time= interval since last meal.*

Time hours	Plasma free fatty acids mEq/litre
14	0.76
38	1.28
62	1.50
86	1.99

therefore appears less likely that the high values can be ascribed to lipase activity in the serum.

Earlier investigations have shown an increase in the cholesterol and phosphatides during starvation (Kartin, Man, Winkler & Peters, 1944; Posborg Petersen, 1952). It was not possiblee to interprete this by the transport of the fat from depots to the liver during starvation (Posborg Petersen, 1952). As yet free fatty acids in plasma have been studied only in association with clearing and postprandial lipemia. Observations made in the present investigation suggest the possibility that fat might be transported in the form of free fatty acids.

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January 1956.

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A TECHNICAL AID IN PREPARATIVE PAPER ELECTROPHORESIS

(Received for publication February 4, 1956)

On fractionation of serum by preparative paper electrophoresis the serum components are diluted by the spread of the proteins in the paper moistened with the buffer. Recovery of the protein solutions in the usual way by eluation with salt solutions results in further dilution. This is a disadvantage in serology, for example, because components of low titer in the individual fractions may no longer be demonstrable after such dilution.

In order to recover the serum components after the electrophoresis without further dilution, the following procedure was used. The preparative electrophoretic fractionation method is described earlier (Laurell, 1955). After the electrophoresis the filter papers were cut transversely into 2 cm wide segments. Each segment of the filter paper was then placed in a perforated plastic container, designed to fit into a centrifuge tube (fig. 1). The tubes with the plastic containers were centrifuged for 20 minutes at 3000 r.p.m. In this way the bulk (about 70 per cent) of the buffer and the protein solution were recovered from the paper.

This method avoids dilution of the serum components by eluation. Eluated fractions can, of course, be re-concentrated, but such a procedure requires time and thereby involves the risk of loss of any labile components of the fractions.

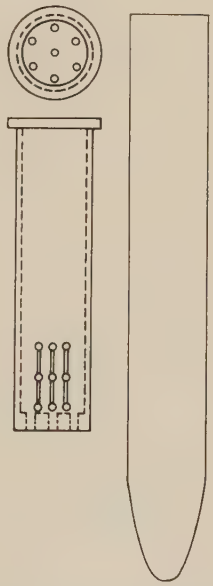

Fig. 1. The perforated plastic container dimensioned to fit into the centrifuge tube.

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Scandinav. J. Clin. & Lab. Investigation 84—85, 8, 1956.

HAS THE ENZYME SYSTEM ACETYLCHOLINE-CHOLINESTERASE ANY SIGNIFICANCE FOR PHYSIOLOGICAL HEMOLYSIS IN THE SPLEEN?

In a milieu of 0.9 per cent NaCl solution with the addition of erythrocytes and acetylcholine I have observed that hemolysis occurs under certain conditions. Due to the presence of cholinesterase in the erythrocytes, the acetic acid splits off from the acetylcholine and the concentration of H^+-ions increases correspondingly, which leads to hemolysis. The lower the pH, the greater the degree of hemolysis. These findings are in agreement with a recent report of Holland & Graham (1955).

Under the conditions employed both by Holland & Graham and the present author, there will be a rapid and relative great decrease in the pH. The effect on the erythrocytes of a slight decrease in pH over a more protracted period will thus not be demonstrable. Therefore further investigations were made in order to study the effect of pH and time independently. I have left the erythrocytes in isotonic acetic acid-sodium acetate buffer, and found that hemolysis may then occur at a relatively slight decrease in pH if the erythrocytes have been left in the milieu for a sufficient length of time. After 10 hours at 39° C hemolysis was demonstrated at pH=6.8, but to obtain corresponding hemolysis after standing for only one hour is was necessary to have a pH under 6.0. Characteristic alterations take place in the erythrocytes before hemolysis; they gradually become spherical.

These in vitro experiments are steps in an investigation on the possible connection between the presence of the enzyme cholinesterase in the red blood corpuscles and the occurrence of acetylcholine in the spleen and the normal hemolysis.

The role of the cholinesterase in the erythrocytes is not known. Nachmansohn states in 1950 that the spleen is rich in acetylcholine, and it is further known that the blood corpuscles remain in the spleen for several hours (Knisely, 1936).

Hemolysis in the spleen, associated with the enzyme system cholinesterase-acetylcholine should, according to the in vitro experiments be recognized by means of a decrease in the pH of the milieu in which the blood corpuscles stay in the spleen.

In order to determine the H^+-ion concentration in blood from the spleen I have developed a micromethod which allows chemical determination of the H^+-ion concentration in a blood volume of 0.02 ml. In a solution containing iodate and iodide, the addition of H^+-ions will free equivalent amount of iodine. The addition of blood corpuscles represents the addition of H^+-ions.

The iodine formed combines with the protein of the corpuscles and is again freed by precipitating the protein with acetone. Thus the concentration of the iodine is a measure of the H^+-ion concentration. The iodine is determined spectrophotometrically in ultraviolet light. By means of this method the pH of blood from the vena cava and from the spleen of rats under light anesthesia was determined. Both blood samples were taken simultaneously. Considerably lower pH values were found in blood from the spleen than in blood from the vena cava (see table).

If the animals had been previously treated with physostigmine so that the cholinesterase activity in the blood corpuscles was markedly reduced, this decrease in pH in blood from the spleen was not found.

pH values of blood from the vena cava and from the spleen of normal rats compared with blood from the vena cava and spleen of rats with inhibited cholinesterase activity in the erythrocytes after pretreatment with physostigmine.

	Full cholinesterase activity			Inhibited cholinesterase activity		
	Venous blood	Speen blood		Venous blood	Spleen blood
Rat No. 1	7,37 7.40	7.22	Rat No. 11	7.37 7.37	7.38 7.43
» 2	7.38	7.24 7.13 7.02	» 12	7.46 7.40	7.38 7.40
» 3	7.38 7.37	7.02	» 13	7.48 7.44	7.39 7.40
» 4	7.42 7.41	7.18 7.10 7.08	» 14	7.38	7.38
» 5	7.43 7.37	7.15 7.12	» 15	7.45	7.37 7.43
» 6	7.46 7.50	7.34	» 16	7.44	7.38
» 7	7.42 7.41	7.05	» 17	7.44	7.37
» 8	7.44 7.46	7.24	» 18	7.44	7.40
» 9	7.37	7.25 7.28	» 19	7.48	7.45 7.37
» 10	7.47	7.05	» 20	7.43	7.42 7.47

The different pH values obtained in spleen blood from the same rat represent blood from different parts of the spleen tissue.

The results of these experiments support the assumption that the spleen affords conditions for hemolysis on the basis of an increased H^+-ion concentration. It is reasonable to believe that the development of this lowered pH may be associated with the splitting of the acetylcholine which has been demonstrated in the spleen, the cholinesterase which is found in the erythrocytes releasing acetic acid, as demonstrated in the first-mentioned *in vitro* experiment.

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March 1956.

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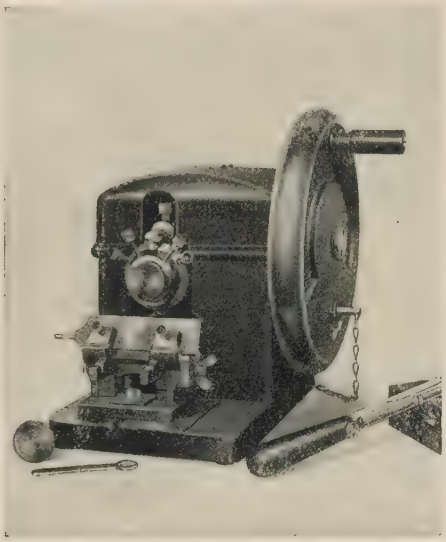


lager 11 forskjellige mikrotomer, med snitt-tykkelse fra minimum 0,1 μ til maksimum 100 μ.

Blant verkets mikrotomer finnes typer for ethvert formål.

Illustrasjonen viser
Seriesnittmikrotom nr. 36, modell Minot

Tekniske data:

Snitt-tykkelse regulerbar fra 1 til 40 μ, i trinn på 1 μ. Objektbord 40 mm, objektklemmenes spennvidde 26 mm, knivens lengde 14 cm.

I Sartorius' hefte „MIKROTOMIE" finner de nyttige vink om mikrotomer og bruken av dem. Skal vi sende Dem et eksemplar?

Vi leverer alt i laboratorieutstyr

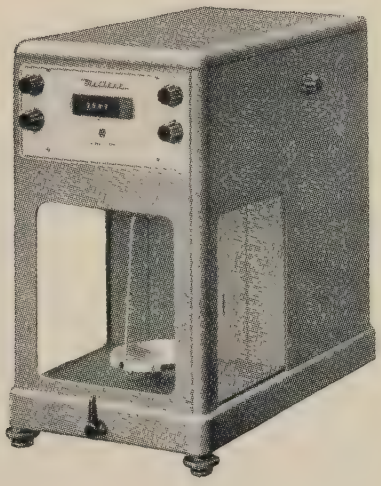
A/s Christian Falchenberg

Oslo *Bergen* *Trondheim*

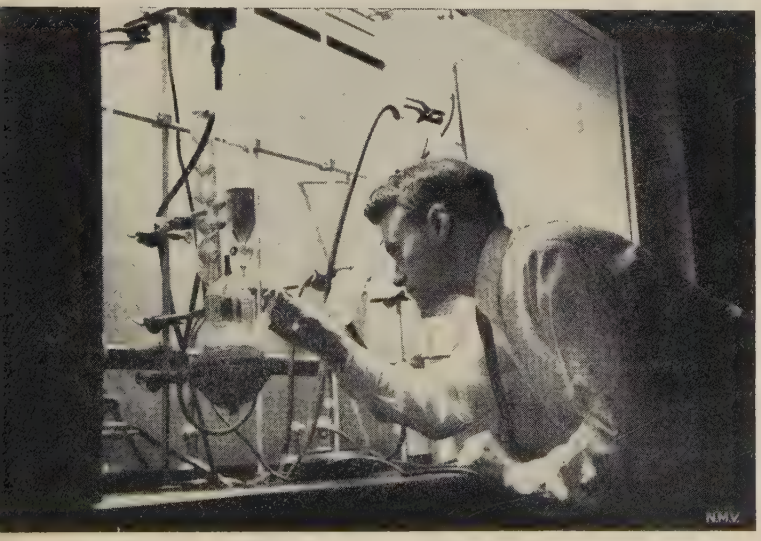
METTLER

ANALYSEVEKTER

og alt til laboratoriet



Type B - 5
Kapasitet: 200 g
Nøyagtighet: ± 0,1 mg



Laboratorieutstyr:

- Elliott volumetriske glassvarer
- Pyrex laboratorieglass
- Unicam spectrofotometer
- Bie & Berntsen van Slyke apparat
- Testa Sicca haemometer

– og alt laboratoriemateriell forøvrig.

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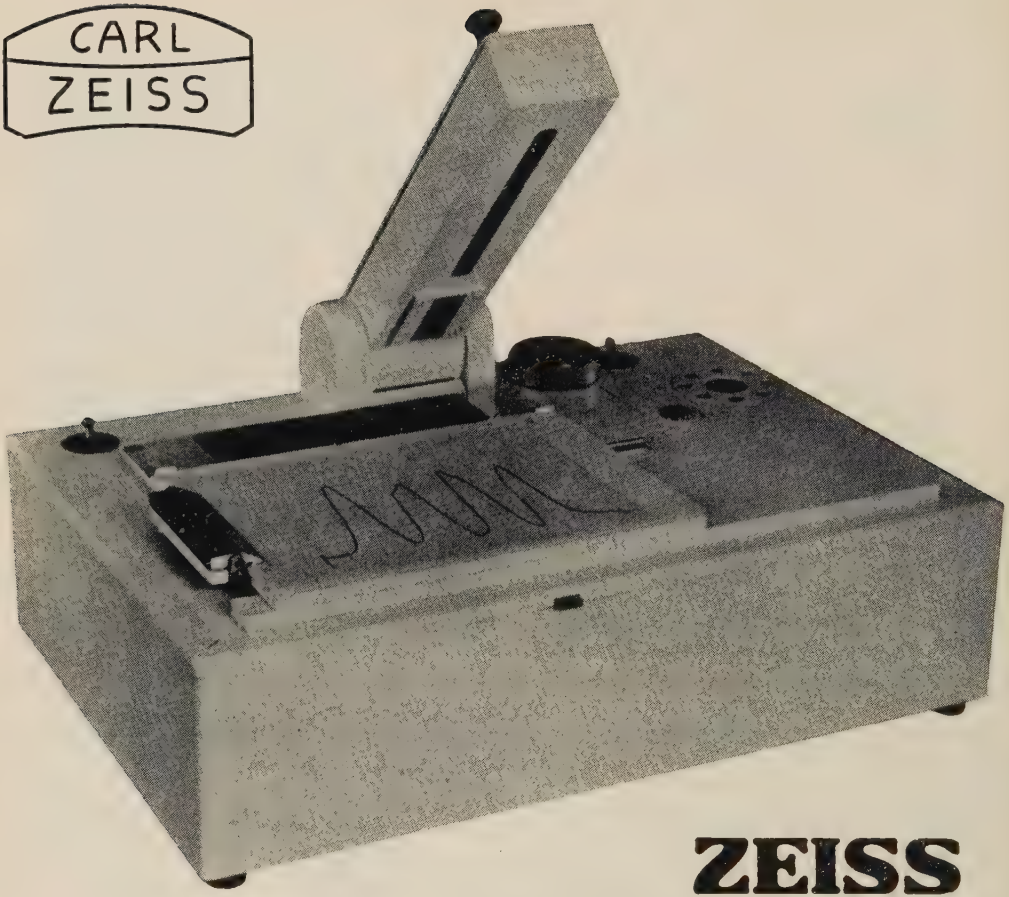

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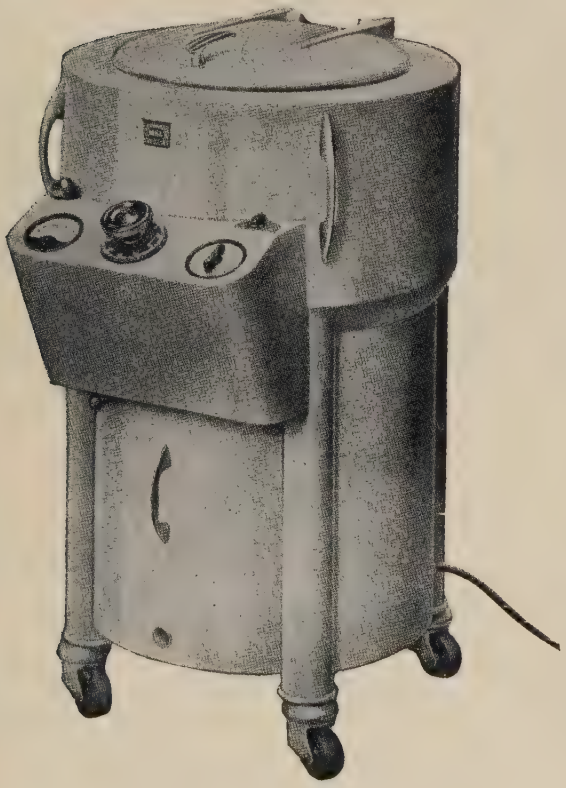

Major: ..... 4000 ml
Magnum: ..... 2400 »
Medium: ..... 1000 »

Frysesentrifuger
Hematocritcentrifuger
High Speed centrifuger

Mikrotomer, frysemikrotomer og Mikrotomslipemaskiner

HISTOKINETTE
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Be om illustrasjoner og tilbud.

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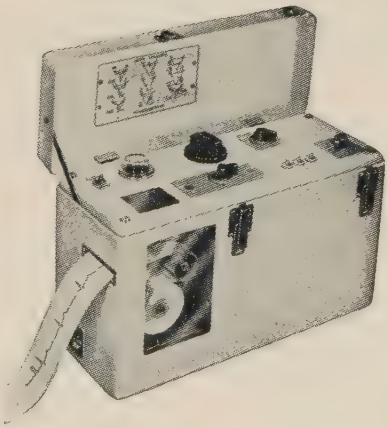

Direkte-skrivende
Elektrocardiografer

MINGOGRAF

Type 12: Een-afleder EKG. Papirbredde: 60 mm. Papirhastigheder: 25-50-100 mm/sek.

Type 21 B: To-afleder EKG til optagelse af een EKG-afledning og een hjertetonekurve. Papirbredde: 60 mm. Papirhastigheder: 25-50-100 mm/sek.

Type 24: To-afleder EKG til optagelse af to vilkårlige EKG-afledninger samtidig eller en blodtrykkskurve samtidig med en EKG-afledning Papirbredde: 100 mm. Papirhastigheder: 5-10-25-50-100 mm/sek.

Type 42: **Fire afleder EKG** til samtidig optagelse af fire vilkårlige EKG-afledninger. Plads for indbygning af hjertetoneforstærker og elektromanometer. Papirbredde: 120 mm. Papirhastigheder: 5-10-25-50-100 mm/sek.

Alle typer er forsynt med »antiforstærker« mod vekselstrømsforstyrrelser.

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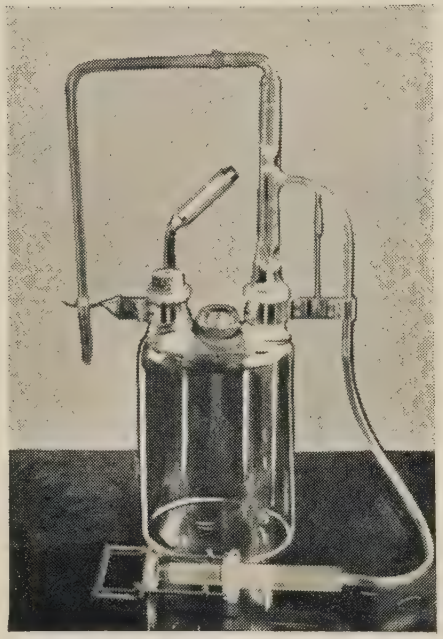

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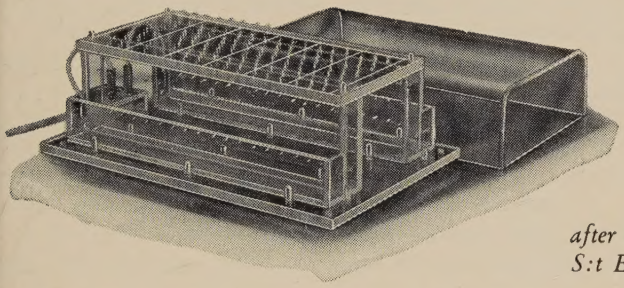
VI[e] Congrès de la Société Européenne d'Hématologie

Copenhagen, Denmark. August 26 — 31, 1957

The provisional subjects of the 6th Congress of the European Society of Haematology will be (1) immunohaematology, blood groups, problems of blood transfusions, (2) serum proteins and their cellular origin, (3) fundamental aspects of haemorrhagic disorders, (4) physiopathology and treatment of leukaemias and related disorders, (5) properdin.

The European Society of Haematology will cooperate with Société Internationale de Transfusion Sanguine. The Congress is open to all physicians and scientists interested in haematology. English, French, and German are official languages.

Dr. J. Bichel, Aarhus, Denmark has the presidency. Inquiries should be directed to the Secretary-General, Dr. Aa. Videbaek, Blegdamsvej 11, Copenhagen Ø, Denmark.



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The introduction should contain a clear statement of the purpose of the investigation and its relation to other works in the same field, but extensive reviews must be avoided.

The experimental procedure must be clearly described. However, methods should be given in extenso only if they represent a new approach.

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